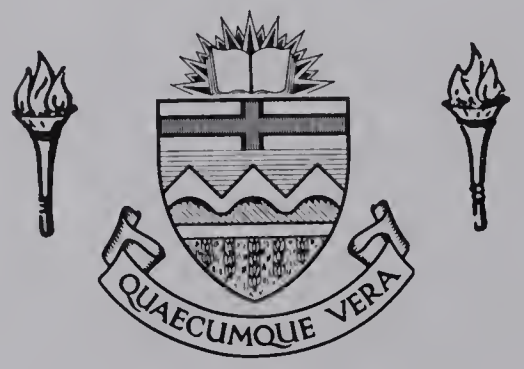

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THE ROLE OF CALCIUM IONS IN EXCITATION-CONTRACTION COUPLING IN MAMMALIAN SKELETAL MUSCLE

by

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A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "The Role of Calcium Ions in Excitation-Contraction Coupling in Mammalian Skeletal Muscle", submitted by William C. Buss in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

Caffeine in concentrations between 2.6 and 20mM readily induced contractures of isolated rat skeletal muscle in oxygenated Krebs solution at 37°C. Contractures could be demonstrated in sodium Krebs buffered with either $NaHCO_3$, or Tris, and in Choline Krebs buffered with $NaHCO_3$. Reduction of the temperature from 37° to 22°C with continuous oxygenation reduced both isotonic KCl- and caffeine-induced contractures. Conditions of 22°C without continuous oxygenation resulted in a rapid elimination of isotonic KCl-induced contractures, but not caffeine-induced contractures. Under these latter conditions contractures were markedly pH dependent; being very small or absent at pH 6.0-6.5 and very large at pH 7.8 and higher. Under the more physiological conditions of continuous oxygenation at 37°C caffeine contractures were also pH dependent, but less markedly so. The pH dependence of muscle caffeine sensitivity is not due to changes in the amount of unionized caffeine in solution, but probably due to an action upon the caffeine receptor or the site of bound Ca^{++} in the muscle. This pH dependence also might reflect a pH dependent membrane passage of caffeine, permitting it to act at internal sites as a function of external pH.

Five divalent cations were tested for their ability to support excitation-contraction coupling in oxygenated

mammalian skeletal muscle at 37°C. Elevated Mg^{++} was not capable of supporting isotonic KCl- induced contractures and exerted a lasting depressent effect on the isotonic KCl response both when added to a solution with Ca^{++} and after the removal of the excess Mg^{++} from the solution. In the presence of Ba^{++} and Ni^{++}, isotonic KCl produced variable, tonic responses. Ba^{++} exerted some lasting depressent effect on contractures even after its removal from the solution, whereas subsequent contractures were potentiated after Ni^{++} was removed from the solution. In the presence of Co^{++}, isotonic KCl-induced contractures were transiently phasic, but soon became tonic. Co^{++} did not suppress contractures when used with Ca^{++}, but it did prevent a clear relaxation in the presence of isotonic KCl. Co^{++} potentiated contractures after its removal from the solution. Sr^{++} was the only ion which demonstrated a clear ability to substitute for Ca^{++}. Sr^{++} maintained phasic, isotonic KCl-induced contractures having maximum tensions well above control responses in the presence of Ca^{++}. Sr^{++} did not greatly affect responses when added to the solution with Ca^{++}, and after its removal appeared to leave subsequent responses relatively unaffected. Sr^{++} also supported caffeine-induced contractures, although less effectively than Ca^{++}, whereas Co^{++} lacked this ability. These divalent cations are thought to act at internal sites of Ca^{++} binding such as the sarcoplasmic reticulum. Sr^{++} is active by itself whereas the others, most clearly Co^{++}, are apparently capable only of various degrees of Ca^{++} displacement from the Ca^{++} binding sites.

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For guidance during the progress of this work, I express gratitude to my supervisor, Dr. G. B. Frank.

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To My Wife, Marodean

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INTRODUCTION

Caffeine has proven to be a useful tool in studies on excitation-contraction coupling in amphibian skeletal muscle. Frank (1961, 1962) has shown that caffeine causes contracture in frog skeletal muscle fibers by releasing Ca^{++} from a binding site in or on the muscle. That this caffeine-mobilized Ca^{++} is identical to the Ca^{++} mobilized by various divalent metallic cations during K^{+}-induced contractures was shown by interaction experiments. These experiments confirm the physiological uniqueness of Ca^{++} in its ability to effect excitation-contraction coupling.

Recently, however, Gutman and Sandow (1965) reported that mammalian muscle is normally insensitive to caffeine-induced contracture. According to this report, chronic denervation is a necessary prerequisite for caffeine contracture in rat skeletal muscle. If this is indeed the case, then by implication there are probably significant differences between amphibian and mammalian muscle systems. This would severely limit the applicability to mammalian muscle of most of the excitation-contraction coupling theory and work which has been done on amphibian muscle.

It was of interest, therefore, to undertake studies on caffeine contracture and the ability of the divalent metallic cations to substitute for Ca^{++} in excitation-contraction coupling in mammalian skeletal muscle.

I LITERATURE REVIEW

I (a) Historical aspects of excitation-contraction coupling

Ringer (1883) was the first to note that it was necessary to bathe frog heart muscle in a medium containing Ca^{++} in order to sustain contractile activity. Locke and Rosenheim (1907) used the capillary electrometer to demonstrate that the spontaneous action current of the rabbit heart remained strong long after the removal of Ca^{++} from the external medium whereas in contrast, the mechanical beat soon became minimal. They concluded that Ca^{++} was necessary for the "production of the wave of contraction out of the wave of excitation".

Mines (1913) used the frog heart to confirm Locke and Rosenheim's observations regarding the dissociation of the electrical and the mechanical responses in a Ca^{++}-free medium. Mines also demonstrated that after the loss of the mechanical response in a Ca^{++}-free perfusion medium, the addition of a Sr^{++} medium greatly prolonged both the electrical and mechanical responses as compared to those in a normal Ca^{++} medium.

The studies of Daly and Clark (1921), and Bogue and Mendez (1930), supported and augmented the observations of Mines; whereas Bay, McLean, and Hastings (1932) observed that the amplitude of the rabbit heart contraction varied directly with the amount of Ca^{++} present in solution.

Heilbrunn (1940, 1943) advanced the thesis that the release of Ca^{++} from the cell cortex led to the coagulation of intracellular muscle proteins, resulting in contraction. Heilbrunn and Wiercinski (1947) provided further evidence for

Heilbrunn's 'Ca^{++} release theory' by injecting various salt solutions via micropipettes into the interior of isolated muscle fibers of the frog. Following length changes microscopically, they found that Ca^{++}, even in dilutions of 0.2mM, caused pronounced shortening. This was the only physiological ion which produced this effect, although Ba^{++} and Sr^{++} also were found to cause shortening.

Heilbrunn and Wiercinski's demonstration of the ability of Ca^{++} to cause contraction was telling evidence against Szent-Györgyi's opposing theory (1945) which had been derived from work done on purified proteins. Szent-Györgyi felt that "since the Ca^{++} and Mg^{++} are bound strongly by myosin and are thus immobilized, it will be the K^{+} which will condition contraction or relaxation by its movement".

Fleckenstein and Hertel (1948) demonstrated that the K^{+}-induced contracture of the frog rectus abdominus muscle could not be well maintained in a Ca^{++}-free medium. The addition of sodium oxalate accentuated this effect.

Sandow, in his classical paper of 1952, pointed out the dependence of contraction on membrane excitation and postulated a process by which a substance released at the surface moved inward to the contractile elements to initiate contraction. Sandow designated the entire sequence of reactions--excitation, inward acting link, and activation of contraction--by the term "excitation-contraction coupling". In line with Heilbrunn's previous proposal, Sandow believed that Ca^{++} played a key role

as the inward acting link. In this paper Sandow provided evidence that longitudinal currents did not play a role in excitation-contraction coupling by reporting experiments in which muscles were massively stimulated. This procedure eliminated local currents and since there was no change in the contractile response, it appeared obvious that local currents were not involved. This conclusion rested on the previous work of Kuffler (1946), who pointed out that a muscle could not develop local currents during K^+-induced contractures because the membrane potential was the same over the entire length of each fiber.

In the intervening years since 1952 a large body of evidence has accumulated in support of Sandow's hypothesis, although some modifications of, or additions to, the details of the process may be necessary. Generally speaking, this evidence rests on certain broad lines of thinking (Fleckenstein, 1964), and these will be treated under separate categories. The discussion in these categories will show that:

a) Ca^{++} is the only physiological ion which will cause contraction when injected into muscle fibers.

b) Ca^{++} influx and efflux are considerably augmented during activity.

c) Variations in extracellular Ca^{++} concentration influence the strength of contractile activity.

d) Ca^{++} has been found necessary for the contractile activity of subcellular muscle substituents.

The discussion of the action of Ca^{++} will be limited, for the purposes of this thesis, to its role in excitation-contraction coupling in skeletal muscle, and to its role in the contractile activity induced by caffeine and the divalent metallic cations.

I (b) Ca^{++} injection and contractile activity

Niedergerke (1955) repeated Heilbrunn and Wiercinski's experiments (1947) of Ca^{++} injection into isolated muscle fibers. Using electrophoretic application, he observed local contraction during the current pulse, followed by partial relaxation when the current was turned off. Niedergerke felt that Ca^{++} was a link in the contractile cycle, but believed that his demonstration provided little evidence for Sandow's thesis of Ca^{++} release and activation since he observed no spread of activity across the fiber.

Caldwell and Walster (1963) cannulated single muscle fibers of the crab and obtained contractions induced by 1-50mM $CaCl_2$, $SrCl_2$, $BaCl_2$ and caffeine. At lower concentrations, the injection of the same molar quantity of $CaCl_2$, $SrCl_2$, $BaCl_2$, and caffeine (at the first injection only), caused contractions of approximately the same duration. Caldwell and Walster suggested that the similarity in the activities of these agents with that of Ca^{++} could be explained by assuming that the former caused the release of Ca^{++} from a site of binding as Frank (1962) had previously proposed. Contractions were either slight or absent, however, when distilled water, KCl, NaCl,

$MgCl_2$, ATP, AMP, EDTA, potassium phosphate, and arginine hydrochloride in concentrations from 10-50mM were injected.

Costantin, Podolsky and Tice (1967) have demonstrated that Ca^{++} induced localized contractions in frog fibers which had been stripped of their sarcolemma (the Natori preparation).

I (c) Ca^{++} influx and efflux during contractile activity

Hodgkin and Keynes (1957) demonstrated an accumulation of Ca^{45} in resting squid giant axon and showed that increased influx occurred with both electrical stimulation and K^+ depolarization. Bianchi and Shanes (1959) found that the Ca^{45} influx in the unstimulated frog sartorius muscle was approximately equal to the squid giant axon's, but that the influx per twitch was approximately 30 times greater in the muscle fibers than in stimulated nerve. During K^+-induced contractures, Ca^{45} entry was even greater than during electrical stimulation, but this entry occurred only at the beginning of the contracture. Substitution of the more polarizable NO_3^- anion for Cl^- led to virtually no increase in resting influx, but a 60% increase during activity with a twitch enhanced by the same percentage. When NO_3^- was substituted for Cl^- in K^+ contracture, there was also a transient increase in Ca^{++} influx and in contracture tension.

While an increased external Ca^{45} concentration led to an increased passive Ca^{45} entry, it did not lead to increased influx with activity, indicating separate membrane sites of entry for the two processes. Qualitatively and quantitatively,

Bianchi and Shanes concluded, it appeared that Ca^{++} entry initiated and controlled contraction. Certainly these findings suggested a special role for Ca^{++} in muscle contraction.

In an analogous study, Shanes and Bianchi (1960) studied the release of Ca^{45} from the frog sartorius during electrical and elevated K^{+} stimulation. They found an accelerated release of Ca^{45}, but only during stimulation and independent of stimulus rate. In unfatigued muscles, the minimum amount of Ca^{45} liberated during an isotonic twitch was approximately equivalent to that which had previously been found to enter during a twitch. The fact that the action potential underlay Ca^{++} release following electrical stimulation was demonstrated by the constancy of Ca^{45} release even under conditions when contraction became progressively weaker. The time course of release during K^{+} contracture, however, was a function of the nature of the contracture, suggesting that the shortening process influenced Ca^{++} release. Nevertheless, the minimal Ca^{45} release during the first 10 minutes of treatment with K^{+} was approximately the same for either isometric or isotonic contracture.

In the guinea pig heart, Winegrad (1960) demonstrated that the increase in the strength of contraction produced by increasing the rate of stimulation was accompanied by a directly proportional increase in Ca^{++} uptake per beat. In contrast, Bianchi and Shanes (1959) demonstrated that Ca^{++} uptake was not increased per twitch in frog skeletal muscle when the frequency of stimulation was increased from 0.5 to 1 per second.

In view of Bianchi and Shanes' demonstration of increased influx of Ca^{45} during activity in the presence of NO_3^- which is associated with an increase in twitch tension, and Winegrad's demonstration of the linearity of Ca^{++} influx and contraction strength, it would appear that there is a quantitative correlation between the strength of contraction and the amount of Ca^{++} entering the cell. In support of this contention is Bianchi and Shanes' demonstration of a temporal correlation between the transient Ca^{++} influx and transient K^+ contracture of fast frog sartorius muscle in spite of continuing depolarization. In confirmation of this temporal correlation, Shanes (1961) predicted and then demonstrated that the sustained contracture of the 'slow' rectus abdominus muscle in elevated K^+ was associated with an elevated and sustained Ca^{++} influx.

Weiss and Bianchi (1965), however, have recently found that frog sartorius muscle has an increase in Ca^{++} uptake in NO_3^- Ringer's vs Cl^- Ringer's at K^+ concentrations between 16 and 32mM, but not at 48 and 80 mM. In contrast, the maximum tension developed and the area under the tension curve increases over the whole range from 16 to 80 mM K^+. Thus Ca^{++} influx does not appear to be quantitatively correlated to changes in tension area curves during K^+ contracture in fast skeletal muscle fibers as it is in twitches.

Evidence was given for strong binding of intracellular Ca^{++} by Hodgkin and Keynes (1957) in squid giant axon, and by Harris (1957), Gilbert and Fenn (1957) and Shanes and Bianchi (1959), in frog muscle. Harris demonstrated that during pro-

longed soaking of frog muscle in Ca^{45}, there was only an exchange of 10-25% of the total Ca^{++} present in the muscle. However, some incorporation of tracer into the "bound" portion was shown by retention of labelled Ca^{++} after exposure to a Ca^{++}-free solution containing EDTA. Since this measurement was done at 4°C, the measurements of Shanes and Bianchi of 38% and Gilbert and Fenn of 39% exchangeable Ca^{++} at room temperature probably represent more accurate figures.

This demonstrated Ca^{++} compartmentalization forms the basis for a division of muscle Ca^{++}. In a simplistic view, there is a 'membrane' or loosely bound Ca^{++} store which is relatively freely exchangeable with external Ca^{++}. The inward flux of this Ca^{++} is associated with contractures induced by agents such as K^{+}. There is also a store of bound Ca^{++}, not as freely exchangeable with external Ca^{++}, and probably localized on the membrane and/or the sarcoplasmic reticulum which can support contracture development under other conditions.

Hill (1948) calculated the time required for diffusion of Ca^{++} from the surface membrane to the vicinity of the contractile proteins and found it to be significantly longer than the time observed between the action potential and the start of mechanical activity. Also, Winegrad (1961), and Frank (1961) have both shown that there exist rather large quantitative discrepancies between the Ca^{++} influx and the amount required for contraction even at the low value of 1 Ca^{++} per myosin molecule. It is further obvious from the calculations of Weber, Herz and Reiss (1963), and Portzehl, Caldwell and Ruegg (1964), that the

threshold Ca^{++} concentration required for activation is larger than that shown by Bianchi and Shanes (1959) to enter during twitch by a factor of 10-100 (Sandow, 1965).

In view of Ca^{++} compartmentalization and the discrepancies that have been demonstrated to exist between the amount of Ca^{++} entering during stimulation and the amount needed at the level of the contractile proteins, special mechanisms must exist for the increased release of Ca^{++} during activity. Even if no net gain or loss of Ca^{++} occurs, it would seem that marked shifts must occur in localized intracellular regions of individual fibers during activity. Influx and efflux might then only reflect these fundamental processes of Ca^{++} mobilation that are involved in contraction and contracture. Bianchi (1961) has suggested that Ca^{++} influx during stimulation may be due to an uncharged Ca-ion pair. Hodgkin and Horowicz (1960) postulated that depolarization initiated contraction by permitting the entry of an activation particle, possibly a negatively charged Ca-ion complex.

Considering these inconsistencies, it appears that certain modifications of Sandow's original hypothesis are in order. One possibility is that in fast skeletal muscle the entry of Ca^{++} is involved in the release of further and larger amounts of Ca^{++} from cellular stores, either from the membrane or from the sarcoplasmic reticulum. In other muscle systems such as cardiac muscle or smooth muscle, Ca^{++} influx from the external membrane may be quantitatively sufficient to cause contraction.

I (d) Variations in extracellular Ca^{++} and contractile activity

In the years prior to Sandow's hypothesis of 1952, few reports had appeared citing the necessity of extracellular Ca^{++} for contractile activity. Since 1952, and particularly in the past few years, Ca^{++} has been demonstrated necessary for virtually all types of muscle tissue and for the action of many drugs and transmitter substances associated with contractile activity. In fact, Ca^{++} has been implicated in such a wide variety of pharmacological responses (e.g. neurotransmitter release, mitrochondrial volume changes, melanophore dispersion), that it has been proposed as the means of stimulus-response coupling: a category which includes as a subcategory excitation-contraction coupling (Dikstein and Sulman, 1966).

Bianchi and Shanes (1959) have demonstrated the necessity of external Ca^{++} in relation to activity influx. Frank (1958, 1960, 1961) has demonstrated that K^{+}-induced contracture of fast frog muscle fibers may be rapidly eliminated in a Ca^{++}-free medium. The use of the K^{+}- induced contracture circumvents the problem of the parallel loss of electrical excitability and contractile activity that occurs in a Ca^{++}-free medium and thus the difficulty of testing the divorcement of membrane excitability and contraction. In contrast, inhibition of K^{+}-induced contractures occurs in a Ca^{++}-free medium without a change in the degree of K^{+}-induced depolarization (Frank, 1958) or in the

resting membrane potential (Frank, 1964).

Frank (1960) demonstrated that the rate-limiting step in the elimination of the K^+-induced contracture in a Ca^{++}-free medium was diffusion of Ca^{++} from the extracellular space, since the muscle size rather than the fiber size determined the length of time required for elimination. This was further validated by the demonstration that EDTA added to the Ca^{++}-free solution speeded K^+-induced contracture elimination. In the same paper (Frank, 1960), evidence was presented which showed that not only was the contracture reduced in a Ca^{++}-free, elevated K^+ solution, but the size of the K^+-induced contracture could be reduced by equilibration with a solution of reduced Ca^{++} content. Thus in 0.05mM Ca^{++} the 25 mM K^+ contracture was reduced by 55%, but even at 0.01mM Ca^{++} a contracture could still be elicited. When the Ca^{++} concentration was doubled to 2.16mM, the K^+-induced contracture was reduced in maximum tension developed, but increased in duration. At Ca^{++} concentrations below the normal 1.08mM, the muscles went through a phase of potentiation before contracture tension fell off consistently in the range from 0.108 to OmM Ca^{++}.

In further work, Frank (1961, 1962) demonstrated that a number of multivalent metallic cations were apparently capable of substituting for Ca^{++} in support of isotonic KCl (123mM)-induced contractures. Thus, after isotonic KCl-induced contractures were eliminated in a Ca^+-free Ringer's solution (normally 1-10 minutes), ions such as Co^{++} and Ni^{++} were capable of supporting isotonic KCl-induced contractures over a further

time course. While the effects of these ions were variable, e.g. Co^{++} and Ni^{++} produced a maximum tension equal to or greater, and Mg^{++} and Sr^{++} less, than that in the presence of Ca^{++}, these ions could not support contractures over the 5 hour or longer time course that Ca^{++} could. Instead, contracture tension fell off rather sharply after 2-3 hours in the presence of the cations other than Ca^{++}. This was not a functional decay, as was shown by the restoration of the contracture with the reintroduction of Ca^{++}. This effect suggested that the metallic cations did not have an effect identical to Ca^{++}, but rather released Ca^{++} from a binding site in or on the muscle. Further evidence for this mechanism will be presented when caffeine is discussed in a following section.

I (e) Contractile activity of subcellular muscle substituents

Bozler (1954) was among the first to point out the dependence of the contractile activity of glycerol extracted muscle fibers on the presence of Ca^{++}. In relaxed fibers, and in the presence of ATP, Ca^{++} caused contraction and EDTA relaxation. Mg^{++}, in the presence of ATP, had two distinct effects; it could cause contraction at low concentrations and a relaxation at higher concentrations which could be overcome by the addition of Ca^{++}. Bozler suggested that the action of the relaxing ('Marsh') factor and of EDTA could be explained similarly; i.e. by the complexing and inactivation of Ca^{++}.

Ebashi (1961) found Ca^{++} necessary for ATPase activity and the superprecipitation of actomyosin, and that the relaxing

activities of several chelating agents had a direct correlation with their Ca^{++} binding activities. Furthermore the ATP dependent Ca^{++} binding activity of the vesicular relaxing factor (presumably part of the endoplasmic reticulum) had a capacity that was comparable to these chelating agents.

Weber and Winicur (1961a, 1961b) pointed out that contractile activity could be demonstrated in actomyosin systems upon the addition of Ca^{++} at a concentration greater than $10^{-5}M$ when ATP, Mg^{++}, and one of several relaxing factors (all of which could bind Ca^{++}) was present. They described two possibilities for Ca^{++} action; 1) Ca^{++} could combine with actomysin and thus initiate contraction, or 2) Ca^{++} could interfere with a reaction between a relaxing factor and actomyosin. In the first instance, relaxation prior to Ca^{++} addition would have been due to the removal of Ca^{++} by the relaxing factor, while in the second instance, contraction inhibition would have been effected by combination of the relaxing factor and actomyosin. Weber and Winicur (1961b) found that the absence of Ca^{++} inhibited superprecipitation only when the Mg^{++} concentration was greater than 0.01mM. This suggested that Ca^{++} caused superprecipitation by blocking the inhibitory effect of Mg^{++}. Weber and Herz (1961) demonstrated that syneresis and maximal ATPase activities of myofibrils required Ca^{++} in a fashion very similar to that of the actomyosin systems.

Weber and Herz (1963) found that Ca^{++} is exchangeably bound to actomyosin and to myofibrils, and that this binding activates ATPase activity and superprecipitation. Ca^{++} binding

and thus ATPase activity and superprecipitation all could be described directly as a function of the Ca^{++} concentration in the medium, but with different functional relationships. Thus if it is assumed that all the myofibrillar Ca^{++} is bound to myosin, ATPase activity becomes half-maximal at 70-80% of the maximum Ca^{++} binding. The amount of Ca^{++} bound to the myofibrils was a function of ionic strength and the concentration of Mg^{++} and ATP in the medium. When the Ca^{++} concentration was raised to 2-10μM, exchangeable Ca^{++} was found to bind to actomysin resulting in maximum ATPase activity and superprecipitation in the ratio of 1-2M Ca^{++} per M of myosin (i.e. 1-2μM Ca^{++} per g. actomyosin). The concentration of greater than 0.5μM Ca^{++} shown by Weber, Herz, and Reiss (1963) to be normally necessary for the initiation of the contractile activity of actomyosin systems correlated well with the intracellular concentrations of 0.3-1.5μM found by Portzehl, Caldwell, and Ruegg (1964) to be necessary for the activation of living muscle fibers. The range of Ca^{++} concentrations for maximum to minimum syneresis in myofibrils was 10^{-6} to 10^{-7}M (Weber, Herz, and Reiss, 1964).

One of the possibilities that Weber et. al., had advanced in the course of their work was that Ca^{++} was required only to overcome the contractile inhibition produced by Mg^{++} and ATP. This thesis was found to be tenable in regards to ATP since at low ATP concentrations partial superprecipitation occurred in the presence of 2mM EGTA, which removed almost all of the Ca^{++}. When the Mg^{++} concentration was lowered, however, the binding of Ca^{++} to myofibrils in the presence of EGTA increased to the

extent that more Ca^{++} was now bound than at high Mg^{++} and without EGTA. Superprecipitation accompanied this increase in Ca^{++} binding. These experiments suggested the existence of 2 or more Ca^{++} binding sites. In the presence of Mg^{++} or Mg-ATP, only one of these sites was saturated with Ca^{++} and thus only half as much Ca^{++} was bound as when Mg^{++} was omitted. Increasing the Ca^{++} concentration did not increase the quantity of bound Ca^{++}. If the concentration of Mg^{++} was lowered, however, additional Ca^{++} could be bound to the second site. In the presence of Mg^{++} or Mg-ATP, most of the bound and exchangeable Ca^{++} was removed by EGTA as shown by complete inhibition of superprecipitation and 90-95% of ATPase activity. Contractile activity, then, appears to be regulated by complex interrelationships between Ca^{++} levels and the concentrations of Mg^{++} and ATP.

Weber, Herz, and Reiss (1964b) found that some actomysin preparations apparently exhibited contractile activity in the presence of EGTA with Ca^{++} concentrations as low as 0.02μM per g. actomyosin. This independence of Ca^{++} and contractile activity may have been a function of the age of the preparation. When the ATP concentration was reduced, partial syneresis occurred around pCa 8 with only 0.2μM exchangeable Ca^{++} bound per g. actomyosin. It could be postulated from these experiments, that relaxation was caused by the binding of 2 ATP per actomyosin, and that Ca^{++} could produce contraction by interfering with the relaxant ATP. Lowering of ATP could then permit syneresis without Ca^{++}. The Ca^{++} insensitive actomyosin, in this concept, would have lost the ability to bind relaxant ATP.

Ebashi (1963) demonstrated that while actomyosin extracted from muscle as actomyosin shows increases in ATPase activity and superprecipitation with ATP and increased Ca^{++} concentration, reconstituted actomyosin does not. He found that it was necessary to add a protein substance, similar to tropomyosin, to restore the actomyosin sensitivity to Ca^{++}. When natural, Ca^{++} sensitive actomyosin was treated with trypsin, which is known to inactivate tropomyosin, the actomyosin was found to be Ca^{++} insensitive. Addition of Ebashi's protein factor to the trypsin treated actomyosin returned Ca^{++} sensitivity. It appears, then, that a tropomyosin like protein may be the actual site of Ca^{++} combination on the contractile proteins.

Weber, Herz, and Reiss (1963) further suggested that the ATPase of actomyosin in the presence of Mg^{++} resulted from the enzymatic action of actin which increases the relatively low rate of ATP hydrolysis characteristic of myosin. Relaxing agents would then prevent actin from activating myosin ATPase without affecting the normally low myosin ATPase activity. If ATP is present in concentrations of greater than 10^{-5}M, a concentration of ATP that normally inhibits hydrolysis and contraction, actomyosin and myofibrils exhibit contractile activity only after the formation of a Ca^{++} complex.

Levy and Ryan (1966) have studied the dependence of the contraction rate of actomyosin on ATP and Mg^{++} concentrations, and have concluded that contraction requires Mg-ATP in a definite, sequential order at two separate sites on the myosin

molecule which has resting links with actin. Only one of these sites hydrolyses ATP. In this view, Ca^{++} overcomes the substrate inhibition of ATP, Mg-ATP is hydrolyzed at one site and then acts at a further site to permit a 'sliding over' where the entire process is repeated.

I (f) Subcellularly bound Ca^{++}

It is evident that since Ca^{++} concentration appears to control contractile activity, there must be some means of controlling Ca^{++} flux. As previously mentioned, the work of Harris (1957), Gilbert and Fenn (1957) and Bianchi and Shanes (1959), gives a basis for compartmentalizing intracellular Ca^{++}, and it could be postulated from these early results that the bound stores of Ca^{++} in muscle could reflect a site of intracellular flux regulation.

Marsh (1952) found that when he replaced the supernatant fraction of muscle homogenates with saline, a 10-fold increase in ATPase activity and contraction of myofibrils occurred. He thus assumed that the supernatant fraction held a relaxing factor, which became known as the Marsh factor. Bendall (1958) found that the relaxing factor was related to the presence of 'granules'. Ebashi and Lipmann (1962) and Muscatello, Andersson-Cedergren, and Azzone (1962) demonstrated that these granules were vesicles of the endoplasmic reticulum or sarcotubular system and exhibited ATP and ATPase dependent Ca^{++} accumulation.

Hasselbach and Makinose (1961,1962) found that the relax-

ing granules of the "endoplasmic reticulum" took up large quantities of Ca^{++} from a solution containing Ca^{++}, Mg^{++}, oxalate, and ATP. In this solution the accumulated Ca^{++} was stored in the granules as the oxalate. In a Ca^{++}-free solution the granules split ATP at a low, "basic" rate, however, when Ca^{++} was added, ATPase splitting or "extra-splitting", increased by a factor of 7-8 times the basic rate. Ca^{++} concentration, activity of the Ca^{++} pump, ATPase "extra-splitting", and a phosphate exchange were all found to be directly correlated. These results suggested that a carrier on the outer surface of the granule membrane became phosphorylated and was thus able to combine with Ca^{++} and transport it to the inner portion of the granule. Weber, Herz, and Reiss (1963) reported that fragments of the sarcoplasmic reticulum were capable of removing virtually all of the exchangeable Ca^{++} bound to actomyosin or myofibrils during the 'contractile' response, and that this removal was directly related to inhibition of contractile activity. It was found that the vesicles were capable of reducing external Ca^{++} to 0.02 µM or less and thus were capable of successfully competing with actomyosin for the Ca^{++} required for contractile activity. Thus the Ca^{++} accumulating ability of the vesicles was sufficient to explain relaxation without invoking any further action of the vesicles on the contractile proteins.

Weber, Herz, and Reiss (1966) found that their evidence suggested that Ca^{++} binding within the sarcoplasmic reticulum

occurred in equilibrium with the internal Ca^{++} concentration. Some of the internal Ca^{++} may have been precipitated as the oxalate. Ca^{++} was found to be the transport system substrate and ATP breakdown was coupled to influx regardless of the net uptake. However, there was a maximal steady state filling which limited flux and ATPase activity. Outflow into low external Ca^{++} was independent of ATP and therefore characteristic of free diffusion.

Hasselbach and Seraydarian (1966) demonstrated that one of three types of sulfhydryl groups, designated Type A, was intimately involved in Ca^{++} transport and ATPase "extra-splitting". These sulfhydryl groups were located on the exterior surface of the membrane of the sarcoplasmic reticulum.

Costantin, Franzini-Armstrong, and Podolsky (1965) used the Natori preparation of denuded muscle fibers to demonstrate the accumulation of externally applied Ca^{++} in the terminal sacs of the sarcoplasmic reticulum. They suggested further, that the proximity of these lateral sacs to the T tubules could implicate them in the Ca^{++} release required for contraction initiation as well.

Winegrad (1965) lent further credence to this suggestion in an elegant experiment using frog muscles labelled with Ca^{45}. Autoradiographs showed localization of Ca^{45} at the level of the lateral sacs in resting muscle. During K^{+}-induced contractures, however, increasing amounts of Ca^{45} moved to the region of actin-myosin overlap as a function of the K^{+} concentration used and the tension produced.

From these studies it is amply clear that the sarcoplasmic reticulum maintains a store of bound Ca^{++}, that its accumulation is very likely involved in the relaxation of muscle fibers, and that it could represent the intimate site for the release of Ca^{++} required for the activation of contractile activity.

I (g) Model of excitation-contraction coupling

In partial summary of the previous discussion, it is now fairly safe to describe a simplified model for the role of Ca^{++} in excitation- contraction coupling in skeletal muscle (Frank 1958, 1961, 1964; Sandow 1965; Nayler 1966). This will be done without the presentation of a detailed histological description.

The initial stimulus consists of depolarization of the surface membrane of the muscle fiber, e.g. that induced by the action potential or by elevated K^{+}. This depolarization of the surface membrane is then transmitted down the T tubules, the membranes of which are continuous with the surface membrane. This depolarization could then permit the displacement of Ca^{++} across the cell membrane, where it could activate myofibrils directly, or, more likely, release intracellularly bound stores of Ca^{++} from the lateral sacs. This triggered Ca^{++} release would then sufficiently raise the myoplasmic concentration of free Ca^{++} to activate actomyosin ATPase and initiate contractile activity.

Alternatively, the signal propagated along the surface and the T Tubule could be transmitted across the junction of the T tubule and its adjacent lateral sacs, increase the permea-

bility of the lateral sacs, and thus cause Ca^{++} release. One possibility for such transmission is via an electrotonic pulse. Ca^{++} would then diffuse out from the lateral sacs under a large concentration gradient and activate the myofibrils. Regardless of the mechanism invoked, however, the role of Ca^{++} is primary. Relaxation is most probably governed by the removal of Ca^{++} from the contractile proteins by the Ca^{++} pump of the sarcoplasmic reticulum.

Contractions elicited by the injection of Ca^{++} into muscle fibers occur regardless of the state of surface membrane depolarization and it is probable that in this case there is a direct activation of the contractile mechanism or an induced further release of Ca^{++} from the sarcoplasmic reticulum. Injected Ca^{++} would then be equivalent to reticularly released Ca^{++}, and the kinetics of contraction and relaxation would be governed by the rate of Ca^{++} uptake by the sarcoplasmic reticulum.

Sandow, Taylor, and Preiser (1965) and Edman, Grieve, and Nilsson (1966) have demonstrated a quantitative relationship between the duration of the action potential and the duration of the active state. This fact is well demonstrated by potentiators such as the divalent metallic cations (Be^{++}, Sr^{++}, Zn^{++}, UO_2^{++}, etc.) which act to prolong the spike and thus cause an increased release of Ca^{++} with its attendant prolongation of the active state and potentiation of the twitch response. Sandow and Isaacson (1966) demonstrated that this potentiation is mediated on the surface membrane due to its rapid obli-

teration by metal binding agents, and possibly occurs via phosphate or imidazole groups. The lyotropic anions, in contrast, lower the mechanical threshold.

Costantin and Podolsky (1966) used the Natori preparation to show both chloride and electrically stimulated contraction of frog fibers devoid of their surface membranes. They interpreted this excitation to indicate that depolarization occurred across the internal membranes at stimulus levels known to depolarize surface membranes. Podolsky and Costantin (1966) have also suggested that a chemical transmitter such as acetylcholine might be liberated from the T tubule area following its depolarization, and this in turn would depolarize the sarcoplasmic reticulum and lead to Ca^{++} release. However, acetylcholine itself applied to the skinned frog muscle fiber was without activity.

It is evident that many details of the processes involved in excitation-contraction coupling are unexplainable at the present time. To mention a few salient problems, one could question the exact mechanism by which the internally bound Ca^{++} is released under physiological conditions, the mechanism by which it is reclaimed by the sarcoplasmic reticulum from the contractile proteins, and whether in addition a soluble relaxing factor is involved in relaxation.

I (h) Caffeine and contractile activity

In 1920 a review appeared by Bock on the use of caffeine in a large number of preparations. Bock pointed out the greater

sensitivity of frog skeletal muscle preparations to caffeine as compared to mammalian preparations; although his review consisted mainly of reports such as the injection of a 2% caffeine solution into the hind-limb circulation of a cat, followed by palpation of the hind limb muscles for rigidity. Gasser (1930), in an extensive review of skeletal muscle contractures, noted the similarity between caffeine and chloroform contractures and the deleterious effects of both. Gasser also cited the myothermic experiments of Hartree and Hill (1924) as evidence that caffeine exerts a continuous stimulation of muscle metabolism.

Bianchi (1961) showed that 5mM caffeine increased the resting Ca^{++} influx approximately 3-fold in both polarized and K^{+}-depolarized frog sartorius fibers. However, caffeine did not increase the rapid and transient increase in Ca^{++} influx that occurred at the beginning of the K^{+}- induced contracture. Ca^{++} outflux with 5mM caffeine, regardless of the external Ca^{++} concentration, was also markedly increased, and to about the same extent as influx in a medium with Ca^{++}. A 3-fold increase in resting Ca^{++} influx could be brought about by a 3-fold increment in extracellular Ca^{++} concentration, but there was not an attendant increase in outflux. Bianchi pointed out that it was not the increased influx that was the basis of caffeine contracture, since caffeine contracture was unmodified in a Ca^{++}-free medium.

In contrast to K^{+}-induced contracture tensions which are a function of external Ca^{++}, and during which a marked transient

increase in Ca^{++} influx occurs followed by a return to low flux levels even with sustained depolarization, caffeine causes a sustained increase in Ca^{++} influx and outflux which is independent of external Ca^{++}concentration.

Caldwell and Walster (1963) demonstrated that the external application of 5mM caffeine to crab fibers caused a sustained contracture, without showing, in the early stages, any significant reduction of the membrane potential. Caffeine contractures could be obtained not only when the fibers were depolarized with K^+, but even during the stage of repolarization induced by transfer from a high to a low K^+ solution. Both $CaCl_2$ and caffeine injected into normally polarized fibers or fibers depolarized by high external K^+ caused contracture.

These results were in direct contrast to the previous report of Axelsson and Thesleff (1958). Axelsson and Thesleff reported that caffeine was capable only of activation when externally applied to frog fibers. They noted that the fiber area which they approached with a micropipette containing 80mM caffeine contracted as they reached the membrane. This was undoubtedly due to leakage from the microelectrode tip into the extracellular fluid, and thus the underlying fibers were already contracted when they achieved internal implantation of the electrode. Since they observed only a slow relaxation of the fibers, they concluded that caffeine could not act intracellularly. In view of the rapidity of caffeine action, this was generally believed to be the case until the report of Caldwell and Walster (1963).

Caldwell and Walster pointed out, however, that any intracellular caffeine action would have been masked by extracellular activation, and that the slow relaxation seen probably represented the weakening of caffeine contracture observed with repeated or prolonged caffeine exposure. This decline in the ability to contract under the influence of caffeine could reflect the ability of caffeine to complex with, and solubilize, organic acids. Caffeine may solubilize organic components of the membrane, leading to functional decline. This conclusion is supported by the evidence of destructive histological changes induced by caffeine observed by Bock (1920), Gasser (1930), and Conway and Sakai (1960).

Axelsson and Thesleff (1958) noted that elevated Ca^{++} or Ca^{++} deficient medium had no effect on the caffeine contracture, and that caffeine acted without changes in the resting membrane potential or alterations in the transverse membrane resistance. Obviously, then, caffeine acted at a site subsequent to membrane depolarization, and membrane depolarization was not a necessity for contractile activity.

The above results are readily explicable if one postulates that caffeine reduces the binding of Ca^{++} in the surface membrane and/or the sarcoplasmic reticulum. This would increase intracellular Ca^{++} which would, in turn, be reflected by an increase in flux from a medium with Ca^{++} or in increased outflux in a Ca^{++}-free medium. Bianchi (1962) has demonstrated that caffeine moves essentially freely through frog muscle, and thus is capable of action at internal sites such as the

sarcoplasmic reticulum.

Nayler (1963) showed that caffeine induced a positive inotrophic response in the toad ventricle muscle. In this preparation caffeine also caused an increased inward and outward Ca^{45} movement and an abolition of the 'staircase' phenomenon. The positive inotrophic activity of caffeine could be directly related to the changed Ca^{++} movement. The loss of the staircase phenomenon can be explained in terms of an increased Ca^{++} permeability of the membrane and sarcoplasmic reticulum, and thus a functional refractoriness to that signal which normally leads to graded increments of Ca^{++} release.

There is further evidence, albeit indirect, that caffeine increases Ca^{++} flux or membrane permeability to Ca^{++}. Mambrini and Benoit (1963) and Delga and Foulhoux (1963) have demonstrated a decurarizing action of caffeine at the neuromuscular junction. This effect can also be explained by increased mobilization of Ca^{++} by caffeine, with an attendant increased release of acetylcholine.

Nagai and Uchida (1960) demonstrated that caffeine contracted fibers relaxed with crude muscle extracts only when relaxing factor was present. Herz and Weber (1965) found that caffeine in concentrations of 8-10mM inhibited Ca^{++} uptake by frog sarcoplasmic reticulum. After maximum Ca^{++} uptake, caffeine could very rapidly release 20-40% of the bound stores of the sarcoplasmic reticulum. At pCa 7 (myofibrillar relaxation), caffeine could release enough Ca^{++} to account for

the caffeine contracture of frog muscle. If, indeed, caffeine increases the reticular membrane permeability to Ca^{++}, then the observed effect would be manifest as an inhibition of the ability of the sarcoplasmic reticulum to accumulate Ca^{++} or a decreased efficiency of the Ca^{++} pump. However, release may involve a critical Ca^{++} level, since Herz and Weber noted that if the reticulum was Ca^{++} filled to half-maximum or less, there was little or no Ca^{++} released by caffeine. Herz and Weber (1965) also noted that caffeine affected the reticulum of rabbit skeletal muscle in a quantitatively similar fashion, in spite of previous reports (see review by Bock, 1920) of the relative refractoriness of mammalian skeletal muscle to caffeine.

Caffeine has been shown to potentiate twitch responses at concentrations of 0.05-4mM and to produce contracture at concentratons of 2-5mM in frog fibers (Axelsson and Thesleff 1958, Frank 1962, Sandow and Brust 1966). Sandow (1965) has also shown that caffeine causes essentially similar changes in the action potential as do the lyotropic anions; a reduction in the mechanical threshold. To a further and smaller extent, caffeine also acts similarly to the divalent metallic cations in prolonging the action potential. These actions of caffeine are also explicable in terms of an increase in intracellular Ca^{++} release. Although the action of both caffeine and the divalent cations undoubtedly results in an increased Ca^{++} release during activation, the ions are believed to act at the surface membrane to mediate changes in the action potential, whereas caffeine is thought to act at an intracellular site. Bianchi (1961) has

shown that subcontracture concentrations of caffeine also prolong K^+-induced contractures, and that this prolongation could be reversed by caffeine washout.

Previous reference has been made to the demonstration by Frank (1961, 1962) that various divalent metallic cations were capable of supporting K^+-induced contractures for a further, but limited, time after contracture elimination in a Ca^{++}-free medium. After K^+-induced contractures supported by a divalent cation in a Ca^{++}-free medium were eliminated, K^+-induced contractures were restored with the reintroduction of Ca^{++}. Subsequently, K^+-induced contractures were again rapidly eliminated in a Ca^{++}-free medium, but again could be supported by the addition of the metallic cations. The implications here are, obviously, that the metallic cations did not act by replacing Ca^{++}, but instead released Ca^{++} from a bound site which was not immediately depleted in a Ca^{++}-free medium, and which was not released by depolarization with K^+. This bound site might be located on the surface membrane and/or on intracellular sites such as the sarcoplasmic reticulum.

Caffeine was found to have a similar action on Ca^{++}. In a Ca++-free medium, caffeine gave contractures for a limited time only. This effect was not a functional decay, although this also occurred to some extent, because the caffeine contracture could be restored by the addition of Ca^{++} to the medium bathing the muscle.Frank confirmed this proposal by showing that depletion of the site by repeated isotonic KCl-induced con-

tractures supported by the divalent cations, in a Ca^{++}-free medium, left the frog skeletal muscle insensitive to caffeine. Similarly, depletion of the binding site by caffeine-induced contractures in a Ca^{++}-free medium, made the muscle fibers insensitive to divalent metallic cation supported K^{+}-induced contractures. Reintroduction of Ca^{++} restored both the K^{+}- and caffeine-induced responses. These findings further substantiate the hypothesis that Ca^{++} plays a unique physiological role in excitation-contraction coupling.

Although not normally present under physiological conditions, Sr^{++}, according to recent evidence, is also capable of supporting excitation- contraction coupling. Edwards, Lorković, and Weber (1966) have recently shown that K^{+}-induced contractures in frog muscle may be supported by Sr^{++} for approximately 5 hours, although Sr^{++} was less effective than Ca^{++}. Sr^{++} was found to restore K^{+}-induced contractures after their loss when supported by Mn^{++}, Co^{++}, or Ni^{++}, in a Ca^{++}-free medium. Caffeine contractures could be partially restored in a Sr^{++} medium after their elimination through repeated caffeine exposure in a Ca^{++}-free medium. These findings were correlated with an increased Sr^{++} uptake during activity, while, in contrast, Co^{++} uptake was unchanged. Sr^{++} could fully activate myofibrillar ATPase and was accumulated by the sarcoplasmic reticulum, although Sr^{++} was not as active as Ca^{++} in these activities. The myofibrillar ATPase had, for example, 30 times more affinity for Ca^{++} than Sr^{++}. The correlation between Sr^{++} activity and its ability to activate ATPase and undergo

accumulation by the sarcoplasmic reticulum suggests that the essential features of the previously proposed model are correct.

Nayler (1965) reported that Sr^{++} is the only ion which substitutes for Ca^{++} in cardiac muscle. Seidel and Gergely (1963) found that rabbit myofibril ATPase, normally activated by Ca^{++}, is also partially activated by Sr^{++}, while Cd^{++}, Co^{++} and Mn^{++} are less effective.

Recently Gutmann and Sandow (1965) have reported that mammalian skeletal muscle does not normally undergo caffeine-induced contractures, even at concentrations up to 20mM. According to their report, chronic denervation is a necessary prerequisite for caffeine-induced contractures in rat skeletal muscle. However, as will be shown in this thesis, a number of unphysiological conditions were used in their experiments which undoubtedly explain the caffeine insensitivity they reported. Buss and Frank (1967) and Frank and Buss (1967) have shown mammalian skeletal muscle to be caffeine sensitive. This is an important point, because if mammalian skeletal muscle is normally incapable of caffeine-induced contractures, then by implication there are probably significant differences in the amphibian and mammalian skeletal muscle systems. It would then be necessary to look, for example, for basic differences in membrane structure which would prevent caffeine passage, or for a different functional relationship between the sarcoplasmic reticulum and the contractile proteins in the two muscle types. This would severely limit the applicability to mammalian muscle of most of the excitation-contraction coupling

work which has been done on amphibian muscle.

Gutmann and Hanzlikova (1966) qualified the previous report of Gutmann and Sandow (1965). They reported that while 'slow' mammalian (rat) muscle, such as the soleus, is normally capable of caffeine contracture regardless of age; the 'fast' mammalian extensor digitorum longus taken from young rats is sensitive to caffeine, but this response to caffeine does not occur in muscles obtained from rats 18-22 or more days old.

It should be noted here that the slow-fast muscle distinction that may be made functionally, histologically, and enzymatically in frog muscle, can not be made as definitely in mammalian muscle (Romanul 1964, Dawson and Romanul 1964). True 'slow' fibers have been shown in the guinea-pig extraocular muscles (Hess, 1961). However, as Gutmann and Hanzlikova (1966) point out, the spread of excitation and speed of shortening criteria used in frog muscles to distinguish 'slow' from 'twitch' fibers are mixed in respect to mammalian muscle fibers. Thus the 'slow' mammalian soleus shows a slow shortening speed but also a propagated action potential (Close, 1964). While there are no distinct histological criteria on which to separate mammalian muscle as to type, this distinction can be made on the basis of the speed of contraction and enzymatic activities.

Rat mammalian muscle does not differentiate into slow and fast types until approximately 2 weeks after birth. This could be an explanation for Gutmann and Hanzlikov's findings (1966). Although there may exist species differences (Gutmann and Hanzlikov did not specify the rat species or varieties

used), this chronological distinction in caffeine sensitivity was not observed in our experiments with fast muscle.

II METHODS AND MATERIALS

II (a) Muscle preparations

The number of mammalian skeletal muscle preparations that have been used and found suitable for excitation-contraction coupling studies is not large. For our purposes, it was necessary to find a predominantly fast skeletal muscle that would exhibit phasic isotonic KCl (IsoKCl) contractures during the period of 4-6 hours required to carry out individual experiments on ion substitution. These requirements put definite limits on the size of the muscle involved. Too large a cross-section results in differential activation of successive fiber layers when the muscle is exposed to IsoKCl (Frank, 1960), resulting in a slow, tonic contracture rather than the phasic one which is characteristic of the individual muscle fibers.

Rat diaphragm strips worked fairly well, but the diaphragm cannot be regarded as a typical skeletal muscle, and such preparations invariably contain large numbers of cut fibers. The anterior gracilis from the rat and the levator ani from 15-30 g. mice were tried without results being obtained, primarily because of the problems of large size and the excessive time required for dissection. Success was achieved with whole muscle preparations of the lumbricale, toe muscles from the rat. Both lumbricales and extensor digitorum longii, larger muscles from the lower leg of the rat, were used for caffeine studies. Essentially the same results were obtained with either muscle.

Male rats of the Sprauge-Dawley strain were used in all experiments. The extensor digitorum longus was excised from rats in the 60-135 g. weight range and the lumbricale from rats in the 130-200 g. weight range. Cross sections of both muscles could be approximated by ellipses with major axis diameters of greater than 2.0 mm (extensor digitorum longii) and less than 1.0 mm (lumbricales). The lumbricales weighed between 1.3 and 3.8 mg and the extensor digitorum longii between 70 and 100 mg.

II (b) Experimental set-up

The muscles were suspended vertically in a 2 cc syringe bath and connected to the anode of a RCA 5734 transducer tube. The muscles were under an original tension of 80-140 mg. All preparations were permitted to equilibrate in the oxygenated bath for at least 30 minutes prior to testing. The temperature of the bath was regulated (37° or 22°C in caffeine experiments, 37°C in ion substitution experiments) by circulating water through a jacket surrounding the muscle bath from a constant temperature unit. Isometric contracture tensions of up to 4 g. were then measured after injection of 10 cc of test solution through an injection port in the bottom of the bath. Suction for drainage was provided at the top of the bath so that the muscle was bathed in solution at all times. Each test was followed by flushing the bath with 30 cc of bathing solution as a wash and a rest period of at least 10 minutes.

In all ion substitution and in some caffeine experiments

the solution in the muscle bath, the bathing, and the test solutions were all oxygenated continuously with 95%O_2-5%CO_2.

Other caffeine experiments were carried out in which only the muscle bath was oxygenated with the gas mixture for 30 minutes prior to the start of testing.

In caffeine experiments in which the pH or temperature was changed, at least 30 minutes were permitted to elapse under the altered conditions before testing with a similarly modified caffeine solution. Solution pH's were determined using a Radiometer 22 pH Meter with aliquots of test and bathing solutions immediately preceding or following individual tests. Responses were recorded on a Recti-riter (Texas Instrument Co.) or on a Type RB Dynograph (Beckman).

II (c) Preparation of the solutions

All solutions were made up in distilled water which had been passed through a deionizer and a filter for the removal of organic material.

1) Normal sodium-Krebs-bicarbonate solution

Individual stocks were used to prepare a solution of the following composition:

Sodium Chloride (NaCl)	119 mM
Potassium Chloride (KCl)	5 mM
Calcium Chloride ($CaCl_2$)	2 mM
Magnesium Sulfate ($MgSO_4$)	1.2 mM
Sodium Bicarbonate ($NaHCO_3$)	25 mM

Glucose	12 mM
D-Tubocurarine Chloride	0.1 g.

A combined sodium bicarbonate and glucose stock was freshly made up whenever solutions were prepared. The pH of the freshly prepared solution was 7.6-7.8. When aerated with 95%O_2-5%CO_2, the pH of the solution dropped to 7.4-7.6. When necessary, a few drops of concentrated HCl were added to adjust the final pH of the solution at 37°C to 7.4 $\pm$ 0.1.

2) Choline-Krebs-bicarbonate solution

Choline-Krebs-bicarbonate solution was prepared by adding 119mM choline chloride in place of the NaCl in the normal solution (normal sodium-Krebs-bicarbonate solution of the above composition). D-tubocurarine chloride was omitted.

3) Sodium-Krebs-Tris solution

Sodium-Krebs-Tris solution contained 25 mM Tris (2-amino-2-(hydroxymethyl)-1,3-propanediol) in place of the sodium bicarbonate in the normal solution.

4) Ca^{++}-free solutions

Solutions without Ca^{++} were made up in the regular fashion with the single omission of 2mM $CaCl_2$. Special care was taken in the preparation of Ca^{++}-free solutions, including their preparation in volumetric flasks used only for this purpose, and the rinsing of all associated glassware used in preparing the solutions in the distilled and filtered water.

5) Isotonic potassium chloride solutions

Isotonic KCl solutions were prepared by dissolving 9.14 g. KCl in 1 liter of solution (123 mM) with or without the addition of 2 mM $CaCl_2$. In the IsoKCl solutions used to test various cations, these cations were added instead of, or in addition to, $CaCl_2$.

6) Caffeine solutions

Caffeine solutions were prepared by dissolving 3 to 20 mM caffeine as the base in 0.5 to 5.0 cc of the Krebs solution with the dropwise addition of concentrated HCl. This solution was then diluted to the desired volume by the addition of more Krebs solution and then buffered with sodium bicarbonate or Tris to the desired pH.

7) Solutions containing divalent metallic cations

Stocks were prepared of the chlorides of Mg^{++}, Co^{++}, Ni^{++}, Sr^{++} and Ba^{++}. Of these ions, Co^{++}, Ni^{++}, and Sr^{++} were added to make a final concentration of 2mM either instead of, or in addition to, 2 mM Ca^{++} in the normal bathing solution. In contrast, 2 mM Ba^{++} was added instead of, or in addition to, 2mM Ca^{++} in a solution in which the 1.2 mM $MgSO_4$ was replaced by 1.2 mM $MgCl_2$. This was necessary to prevent the precipitation of $BaSO_4$.

Since the normal bathing solution contained 1.2 mM Mg^{++}, this cation was omitted in the Ca^{++}-Mg^{++}-free solution. In solutions containing Mg^{++} for testing the effect of this cation, 2 mM $MgCl_2$ was added to the regular 1.2 mM to make a total of

3.2mM Mg^{++}, and the solutions with Ca^{++} and elevated Mg^{++} contained 2mM Ca^{++} and 3.2 mM Mg^{++}.

Although other cations were not tested for their ability to support excitation-contraction coupling, some solubility tests were done. At room temperature, Cd^{++}, Be^{++}, Fe^{++}, Sn^{++}, and Zn^{++} formed precipitates in either sodium-Krebs-bicarbonate or sodium-Krebs-Tris ($MgCl_2$ replacing $MgSO_4$ in both solutions). Cu^{++} was insoluble in sodium-Krebs($MgCl_2$)- bicarbonate solution, but soluble in sodium-Krebs($MgCl_2$)-Tris solution with a color change from blue to purple. It should be pointed out that Tris is known to complex with a large number of the above cations (Hanlon, Watt, and Westhead, 1966). While the use of $MgCl_2$ in place of $MgSO_4$, and Tris in place of bicarbonate circumvents the production of sulfate and carbonate precipitates, it is obvious that further solution modifications would be necessary before attempting to test these cations.

II (d) General experimental approach

In the caffeine experiments, contractures were obtained by bathing the preparation in test solutions having various caffeine concentrations, pH's and temperatures, with or without continuous oxygenation.

In experiments of ion substitution, IsoKCl-induced contractures were obtained in normal Krebs (sodium-Krebs-bicarbonate), and these were followed by contracture elimination by soaking the muscle in a Ca^{++}-free Krebs solution and testing with a Ca^{++}-free IsoKCl solution. The test cation was then

introduced in a bathing solution in which it replaced Ca^{++} and tests were performed using IsoKCl with the added test cation. Next a Krebs solution containing both Ca^{++} and the test cation was introduced into the muscle bath and tests were performed using IsoKCl with Ca^{++} and the test cation. Finally, normal Krebs bathing solution was applied and contractures obtained with an IsoKCl containing Ca^{++}.

In a few cases, control responses were first obtained and then Krebs solution containing both Ca^{++} and the test cation was introduced as the bathing solution and contractures were obtained with IsoKCl containing Ca^{++} and the test cation. The preparation was then returned to a normal sodium Krebs solution. In other cases, the control responses were followed by bathing the preparation in a Ca^{++}-free Krebs solution with the test cation and contractures obtained with Ca^{++}-free IsoKCl containing the test cation.

In some experiments with Co^{++} or Sr^{++}, after control responses were obtained the muscles were exposed to a Ca^{++}-free Krebs solution and tested with a Ca^{++}-free caffeine solution every 10 minutes until a caffeine contracture could no longer be obtained. Next the muscles were exposed to a Ca^{++}-free Krebs solution containing Co^{++} or Sr^{++} and then tested every 10 minutes with IsoKCl containing the same cation. In analogous experiments the muscles were kept in a Ca^{++}-free Krebs solution containing either Co^{++} or Sr^{++} and tested every 10 minutes with IsoKCl until a contracture could no longer be obtained. This was followed by a test with a Ca^{++}-free caffeine Krebs solution.

III RESULTS

III (A) Caffeine contractures

III (a) Control caffeine contractures

Caffeine, in concentrations between 2.6 and 20mM, consistently induced contractures of rat skeletal muscles. Caffeine contractures were studied in both the extensor digitorum longus and the lumbricale. The results were essentially the same in both muscles. These contractures were not only obtained under conditions usually considered suitable for isolated mammalian tissues, i.e. experiments done with the muscle maintained at 37°C in Krebs solution continuously oxygenated with 95%O_2-5%CO_2, but also when choline chloride replaced sodium chloride in the Krebs solution or when a Tris buffer was used instead of the usual sodium bicarbonate buffer.

At 37°C and with continuous oxygenation, caffeine-induced responses were as large as, and generally much larger than, isotonic KCl-induced contractures. In the lumbricales (cross-sectional diameters less than 1.0 mm), the tensions produced during caffeine contractures were generally as large as the maximum tetanic tensions. In extensor digitorum longii (cross-sectional diameters greater than 2.0 mm), the contracture tensions were less, but were still at least 15% or more of the maximum tetanic tensions. Caffeine contractures were graded in tension development according to the caffeine concentration used. Contractures could be obtained for several hours when tests were performed every 10 minutes, although the tension produced by a fixed caffeine concentration gradually decreased over this period.

Caffeine was found to be less effective or virtually ineffective when dissolved (with difficulty) directly in the Krebs solution without concentrated HCl added to aid solution.

III (b) Effects of temperature and oxygenation changes on caffeine contractures

In a series of experiments, conditions were varied from 37°C and continuous oxygenation to the 22°C and the pretest 30 minute oxygenation which had been used by Gutmann and Sandow (1965).

When the temperature was reduced from 37°C to 22°C, with continuous oxygenation with 95%O_2-5%CO_2 in both cases, it was found that both isotonic KCl, and caffeine-induced contractures were greatly reduced but still present. This effect is demonstrated in the lumbricale in Figure 1, using 5mM caffeine. If the gas supply to the bath was turned off after 30 minutes with a bath temperature of 22°C, isotonic KCl-induced contractures rapidly diminished to a very small size or disappeared completely. In contrast, caffeine-induced contractures persisted for up to 6 hours of testing; although the contracture tension did gradually decrease.

III (c) Effects of pH on caffeine contractures at 22°C, 30 minute oxygenation

Further experimentation at 22°C and pretest 30 minute oxygenation demonstrated that the sensitivity of our preparations was strongly influenced by changes in pH. Figure 2 demonstrates pH effects over the range of pH values from 6.0 to 9.0 using 20mM caffeine as the test solution in sodium-Krebs buffered

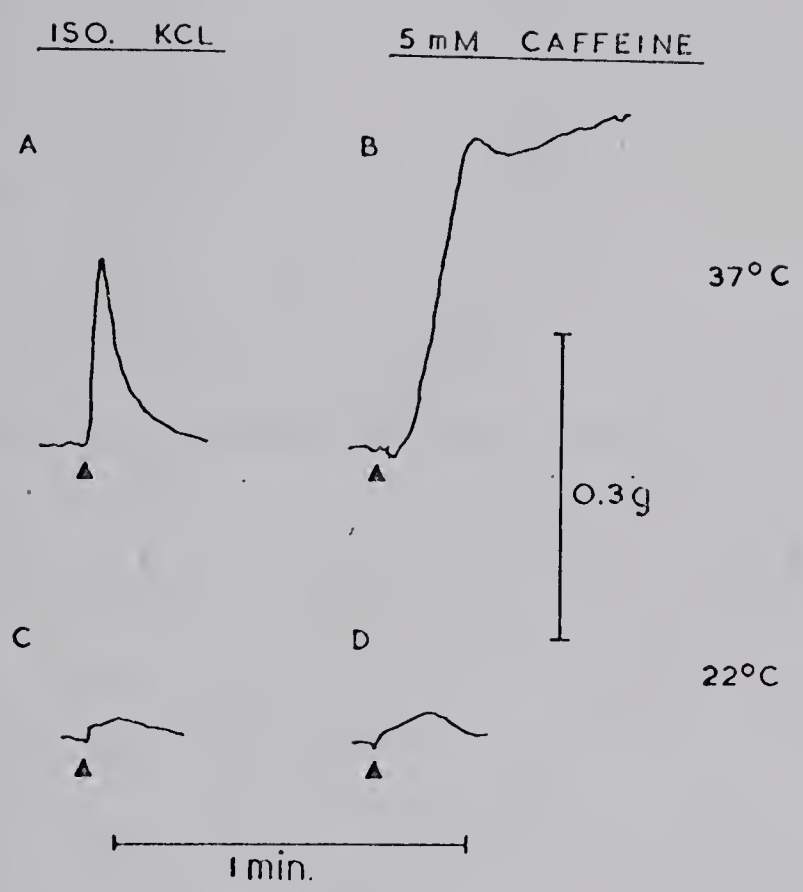

Fig. (1). Effects of reducing temperature from 37° to 22°C on isotonic KCl- (A), and 5mM caffeine-induced (B) contractures of the rat lumbricalc muscle. A and C, isotonic KCl-induced contractures; A, at 37°C; and C at 22°C. B and D, 5mM caffeine-induced contractures; B, at 37°C; and D at 22°C. Preparation was continuously oxygenated with 95% O_2-5% CO_2.

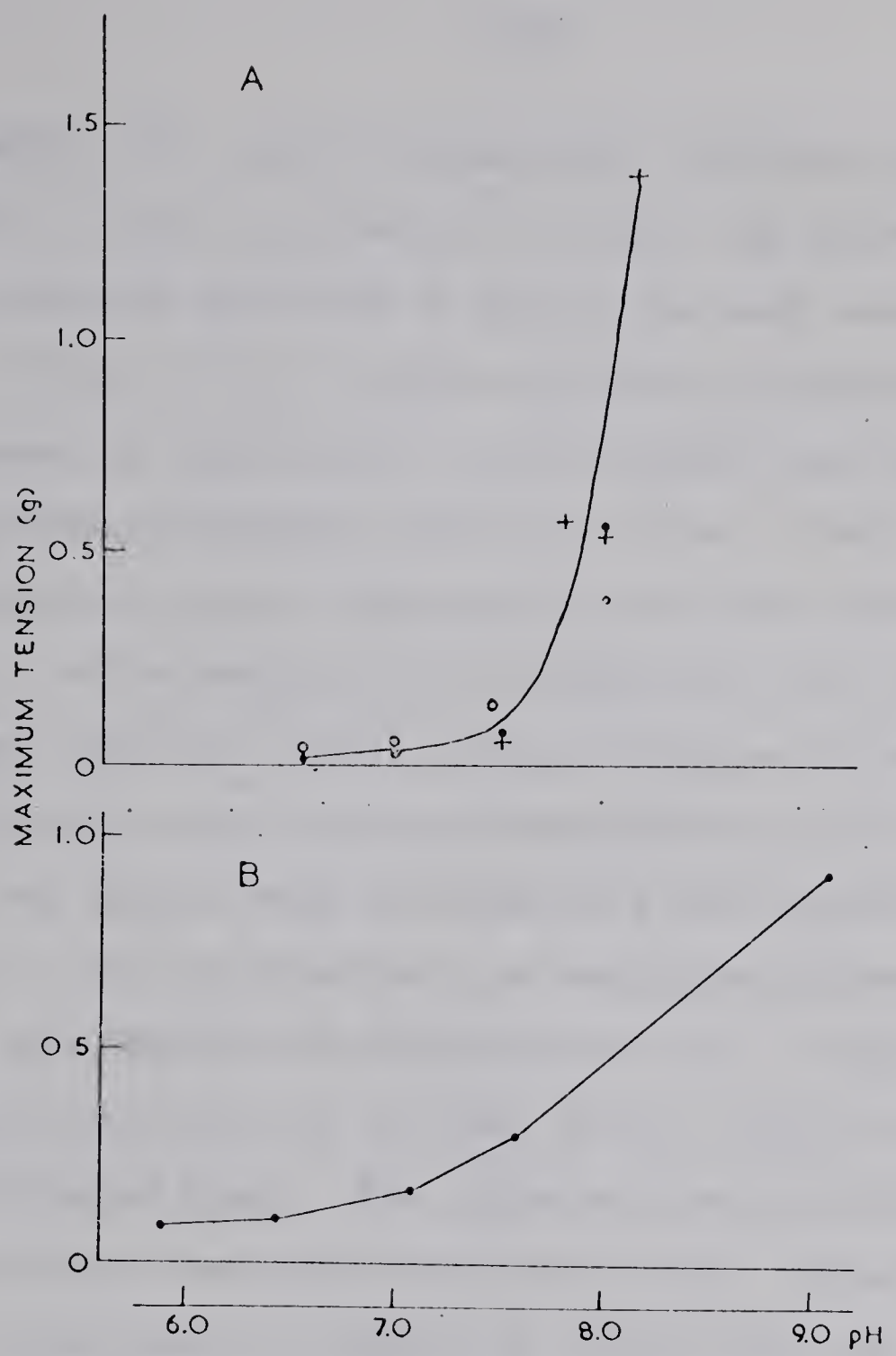

Fig. (2). Effect of changing the pH on contractures induced in the isolated extensor digitorum longus of the rat by 20mM caffeine. A, solutions buffered with $NaHCO_3$; and B, solutions buffered with Tris. A, obtained from three separate experiments with a different symbol used for each preparation; B, obtained from a separate experiment with a different muscle. All preparations kept at 22°C without continuous oxygenation throughout the experiments. All preparations were kept in a bath bubbled with 95%O_2-5%CO_2 for 30 minutes prior to testing.

with $NaHCO_3$ (A), and in sodium-Krebs buffered with Tris (B). While both curves are similar in shape, the curve for the $NaHCO_3$-buffered solutions is seen to be much steeper, breaking sharply at pH 7.6-7.8. Caffeine-induced contractures tended to disappear at pH 6.0-6.5. At pH values just above 7.9-8.0 the bicarbonate-buffered solutions became cloudy, the preparation exhibited a very large caffeine-induced contracture (0.5 to 5 g.), and subsequently the preparation was only capable of producing relatively small, tonic caffeine-induced contractures. This difficulty was not encountered using the Tris buffering system, and points were obtained on a more gradual curve up to pH 9.0. The pH dependence of caffeine-induced contracture tension development was demonstrated over a range of caffeine concentrations from 2.6 to 20mM, and in either sodium or choline, $NaHCO_3$-buffered Krebs. The response size was determined both by the caffeine concentration and the pH. While the absolute tension development at higher pH values was less at lower caffeine concentrations, the abrupt rise in response size tended to occur at approximately 7.6 to 7.8 pH regardless of the caffeine concentration used.

These pH effects were generally demonstrated regardless of the sequence of pH values followed. Occasionally there was a large response to the first test given, regardless of the pH. Repeated testing at a fixed caffeine concentration and pH resulted in reduced contracture tensions, which gradually approached a relatively stable value.

When control solutions of Krebs containing excess $NaHCO_3$ or excess HCl were added, they were without significant effect. Krebs with excess $NaHCO_3$ did elicit a response, however, if the preparation had been tested at some previous time with caffeine. This suggests that sufficient caffeine remained in the tissue to be effective at higher pH's. This is compatible with Bianchi's finding (1962) that frog skeletal muscle was capable of accumulating caffeine to some extent.

In the extensor digitorum longus, low caffeine concentrations and caffeine solutions with low pH values tended to produce tonic responses, while high caffeine concentrations and caffeine solutions with high pH values gave phasic or phasic-tonic responses. The lumbricale generally gave more tonic-like responses regardless of the caffeine concentration or the change in pH.

III (d) Effects of pH on caffeine contractures at 37°C with continuous oxygenation

Further experiments indicated that there was a pH dependence for caffeine-induced tension development at 37°C with continuous oxygenation, but that this pH effect was not as pronounced as at 22°C with 30 minute pretest oxygenation. Thus contractures at pH 6.0 - 6.5 were smaller than those at pH 7.8-8.0 but they were still present at the lower pH, and caffeine contractures at pH 8.0 and 37°C (with oxygenation) were larger than those of the same pH at 22°C without continuous oxygenation. Generally it was necessary to test at the extreme values of the pH range consecutively in order to show clear pH effects. In these experiments pH effects were much more variable, and the muscles often showed an initial large response to caffeine followed by a decline in response size which was not affected by pH changes. In some experiments there was an initial large response produced by each pH change. This initial response was poorly correlated with pH. These initial responses could often be followed, however, by further responses at the given

pH which were more reliably a function of pH. The effects of pH could be shown most clearly by changing the pH during the caffeine contracture (see below--Section (e)).

III (e) Effects of changing the pH during caffeine contractures

In the previous experiments the preparation was allowed to rest for at least 30 minutes in a solution at the pH being tested before applying caffeine in a solution of the same pH. However, it was also possible to obtain essentially the same results using a constant bathing solution pH and using test solutions with different pH's. Thus an almost instantaneous change of pH was effective in demonstrating the pH effects. Changing pH during the course of a caffeine contracture also led to effects ascribable to the new pH value. This is demonstrated in Figure 3 using 20mM caffeine at pH's 6.4 and 7.9 in the extensor digitorum longus oxygenated continuously at 37°C.

III (f) Effects of pH changes on isotonic KCl contractures

In order to determine if the pH effects were due to an action upon the contractile mechanism itself, Krebs solutions of various pH's were used in tests with isotonic KCl. In contrast to caffeine contractures, no consistent pH effects were demonstrated on KCl contractures at 37° or 22°C. This is demonstrated in the experiment shown in Figures 4 and 5 at 37° and 22°C respectively. A double scale at the top of the diagrams gives the pH's of both the test solution (isotonic KCl) and the bathing solution (Krebs). The temperature used is also given at the top of each figure.

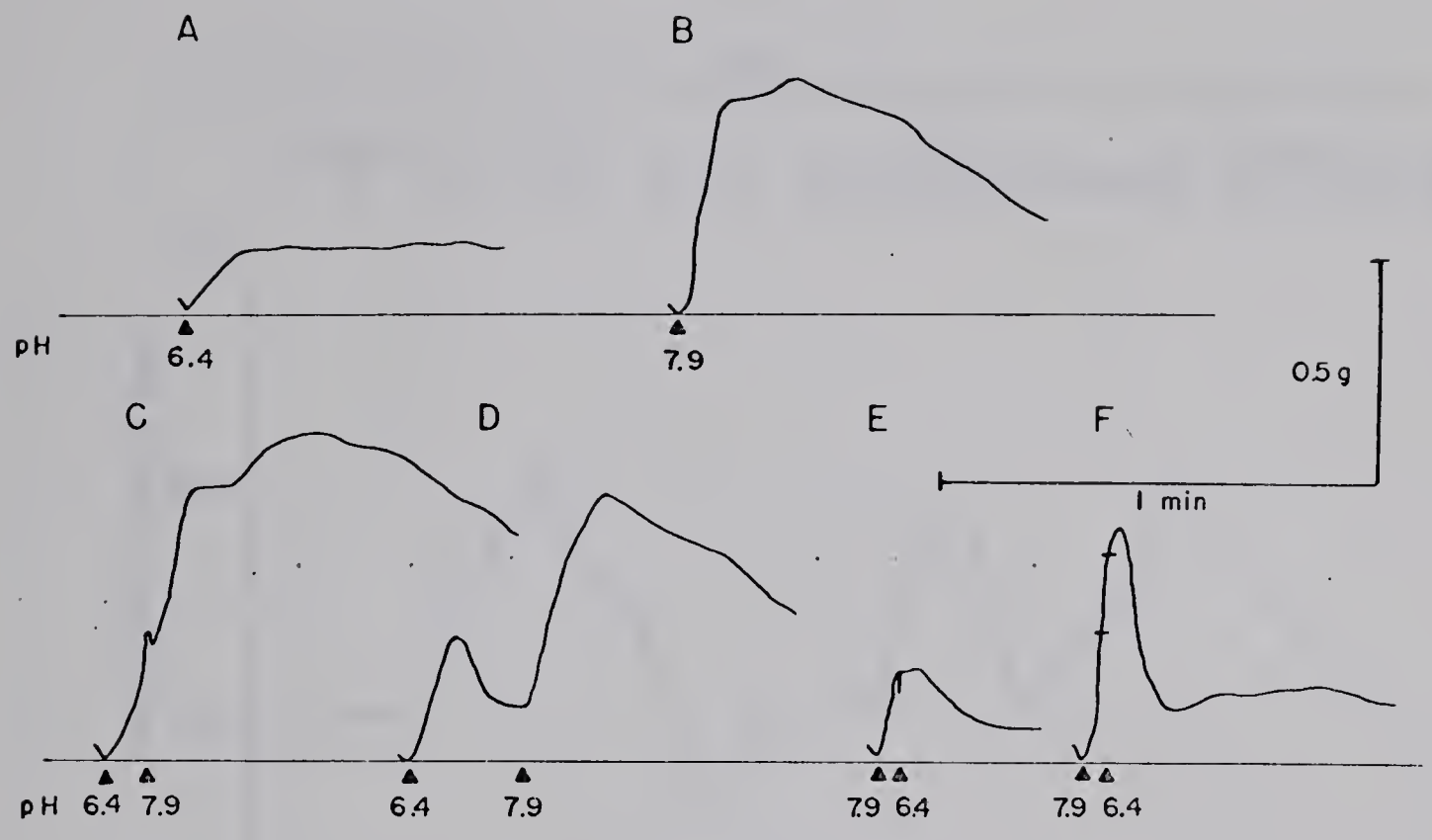

Fig. (3). Effects of changing the pH during contracture induced by 20mM caffeine in the extensor digitorum longus of the rat. A and B, control caffeine-induced contractures; A, pH 6.4; B, pH 7.9. C-F, effects of pH change during contracture: C, pH change from 6.4 to 7.9 immediately after contracture initiation; D, similar change at a later time. E, pH change from 7.9 to 6.4 immediately after contracture initiation; F, similar change at a later time (pH 6.4 solution introduced between two bars shown in F). Preparation kept at 37°C in a bathing Krebs of pH 7.8 continuously oxygenated with 95%O_2-5%CO_2.

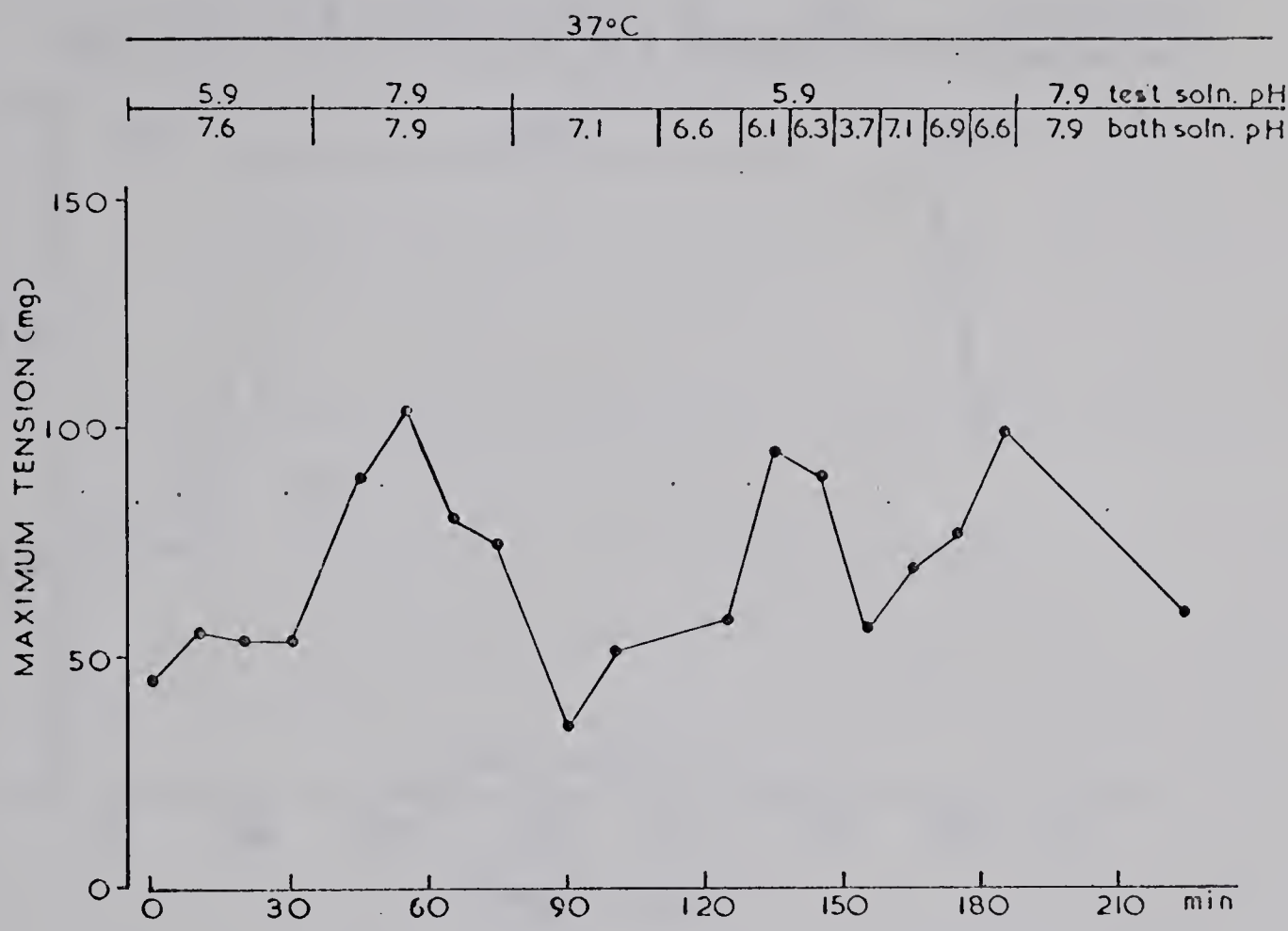

Fig. (4). Effects of changing the pH on isotonic KCl-induced contractures of the rat lumbricale muscle. Scale at top gives the pH of test solution (isotonic KCl) and bathing solution (Krebs). Preparation maintained at 37°C in Krebs solution continuously oxygenated with 95% O_2-5% CO_2. Values of pH adjusted with $NaHCO_3$ or concentrated HCl.

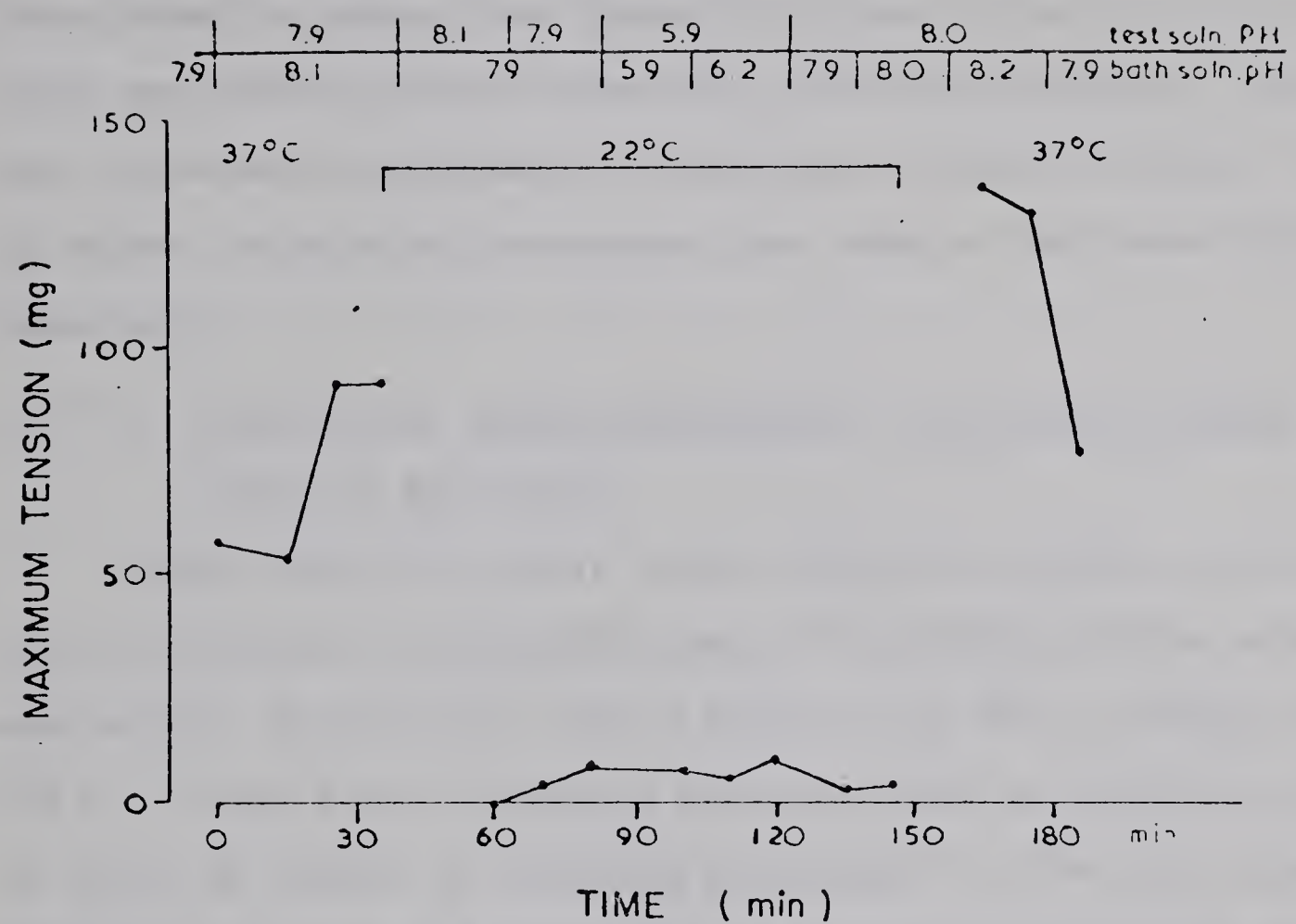

Fig. (5). Effects of changing the pH on isotonic KCl-induced contractures of the rat lumbricale muscle. Continuation of the experiment shown in Fig. 4. Scale at top gives the pH of test solution (isotonic KCl) and bathing solution (Krebs). Preparation temperature changed from 37° to 22°C in Krebs solution continuously oxygenated with 95% O_2-5% CO_2. Values of pH adjusted with $NaHCO_3$ or concentrated HCl.

There was no consistent change of response shape with changes in pH until values above 8.0 were reached. Beyond this point, contractures rapidly dininished in size and were transformed in shape from phasic to tonic. When the preparation was moved from an alkaline to an acidic medium, there was a spontaneous increase in tension, and when changing from an acidic to an alkaline medium, the tension decreased spontaneously.

III (g) Ultraviolet spectrophotometric absorption curves of caffeine solutions

Turner and Osol (1949) found caffeine to have a characteristic ultraviolet spectrophotometric absorption curve between wavelengths 230-300 mu, with a peak at 246 and a trough near 275 mu. When 1/200 dilutions were prepared of caffeine solutions in which an amount of caffeine equivalent to 20mM was dissolved directly in water and with the aid of concentrated HCl and then buffered with $NaHCO_3$, the ultraviolet absorption curves showed no qualitative shifts in the characteristic peaks and troughs. Since the acid-dissolved $NaHCO_3$ buffered and directly dissolved caffeine absorption curves were the same, and were comparable to those shown by Turner and Osol (1949) for caffeine, it may be assumed that acid treatment does not affect the caffeine molecule.

When the ultraviolet absorption curve of a 1/200 dilution of caffeine in a 20mM amount dissolved directly in Krebs was compared with the absorption curve of a 1/200 dilution of caffeine in a 20mM amount dissolved in Krebs with concentrated HCl and buffered with

$NaHCO_3$, a 3% decrease in transmittance was demonstrated with the solution in which caffeine was directly dissolved (Figure 6). While the addition of HCl may facilitate caffeine solubility, this is not borne out by the increase in percent transmittance or apparent decreased caffeine concentration when compared to directly dissolved caffeine. This is an effect that can be repeated and which is of a larger magnitude than can be accounted for by dilution errors. This concentration difference is probably spuriously low due to the dilutions necessary to obtain the absorption curves, since caffeine is known to associate as a function of concentration (Guttman and Higuchi, 1959; see IV--Discussion). While the directly dissolved caffeine may exist to a larger extent in an associated form, these aggregates do not reach colloidal size as was shown by the lack of a visible light beam path through the solution (the Tyndall effect).

Turner and Osol (1949) have demonstrated a plot of pH vs optical density at 240 mu for caffeine. The curve demonstrates a straight line from pH 2 to its termination at 12.5, indicating that caffeine does not react as an acid. Inspection of the curve in the acidic region, however, leads to the conclusion that caffeine has a pK_a of less than 1, confirming the report of Schanker et. al., (1957) of 0.8 as the pK_a.

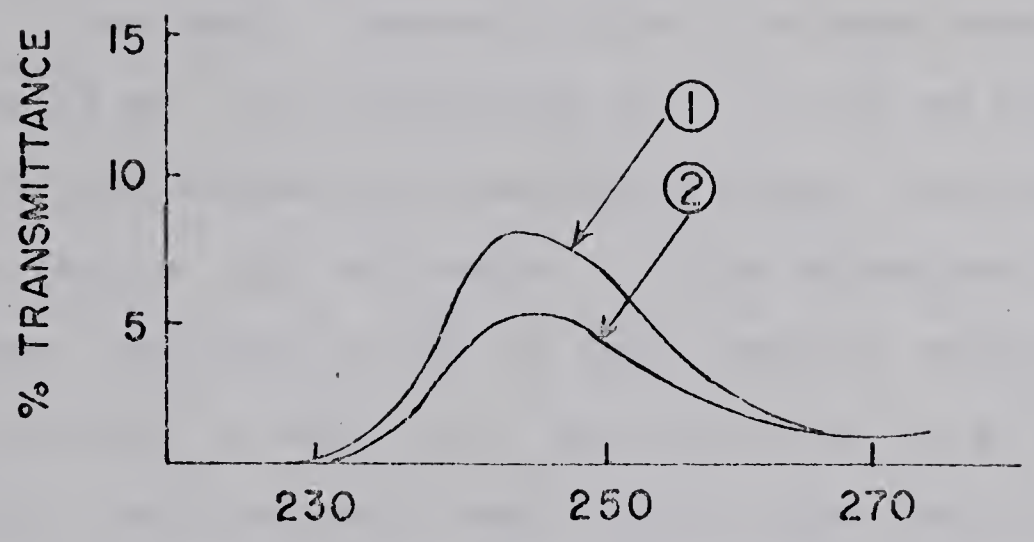

Fig. (6). Ultraviolet spectrophotometric absorption curves for 1/200 dilutions with Krebs solution of 20mM caffeine, pH 7.9. 1; 20mM caffeine dissolved in Krebs solution with concentrated HCl and buffered with $NaHCO_3$. 2; 20mM caffeine dissolved directly in Krebs solution.

III (B) Ion substitution experiments

III (a) Control isotonic KCl contractures and their reversible elimination in a Ca^{++}-free Krebs

Control Ca^{++} IsoKCl responses in rat lumbricales were variable in size and somewhat variable in shape from one preparation to the next. Generally their maximum tensions were about 100-150 mg., but occasionally they were as high as 200-300 mg. A good preparation exhibited phasic contractures in a regular fashion for 4-6 hours. In one experiment, IsoKCl contractures remained at 50% of their control tension after 7 hours of testing in spite of 6 periods of up to 45 minutes in a Ca^{++}-free Krebs (without test during these periods, however). Good IsoKCl-induced responses after 5 hours of experimentation in choline Krebs have been observed. IsoKCl-induced responses declined gradually in time, but often remained quite stable over the period of an hour or two. In a Ca^{++}-free medium, IsoKCl responses were eliminated most often within 20 minutes, but occasionally as long as 40-50 minutes were required for complete elimination.

Caffeine at 2.6mM, under physiological conditions, elicited contractures with approximately the same maximum tensions as IsoKCl. IsoKCl contractures were often potentiated for a test or two following a caffeine contracture, and if a few caffeine responses were obtained prior to IsoKCl contracture elimination in a Ca^{++}-free medium, then the time required for contracture elimination was increased. Repeated caffeine contractures,

however, often made the preparation less sensitive or insensitive to both lower caffeine concentrations and IsoKCl.

The effects on IsoKCl-induced contractures upon exposing rat lumbricale muscles to Ca^{++}-free solutions are shown in Figs. 7 and 8. In the tests shown in Fig. 7, the IsoKCl-induced contractures were eliminated after a 20 or 30 minute exposure to the Ca^{++}-free solution. Also shown is that increasing the duration of the exposure to a Ca^{++}-free solution up to 110 minutes did not consistently modify the time course of the recovery of the contracture response when the muscles were reexposed to a solution containing Ca^{++}. There was some variability in the recovery time course in various muscles, for example recovery was considerably slower in curves A and C than in B and D, but this effect did not appear to be related to the duration of the exposure to the Ca^{++}-free solution.

Although occasionally a preparation would recover to 100% or more of its initial control, Fig. 7 illustrates that in most preparationsthe maximum recovery height was 60-70% of the pre Ca^{++}-free Krebs exposure controls. If the recovery data for curve A is plotted as a % of the maximum contracture tension at 100 minutes (dotted line), then it is seen that the contracture recovery time course more closely approaches a mirror image of the time course for contracture elimination. Thus the slower recovery courses may be viewed partially as an artefact of plotting. Nevertheless, the recovery slopes are still somewhat lower than elimination slopes.

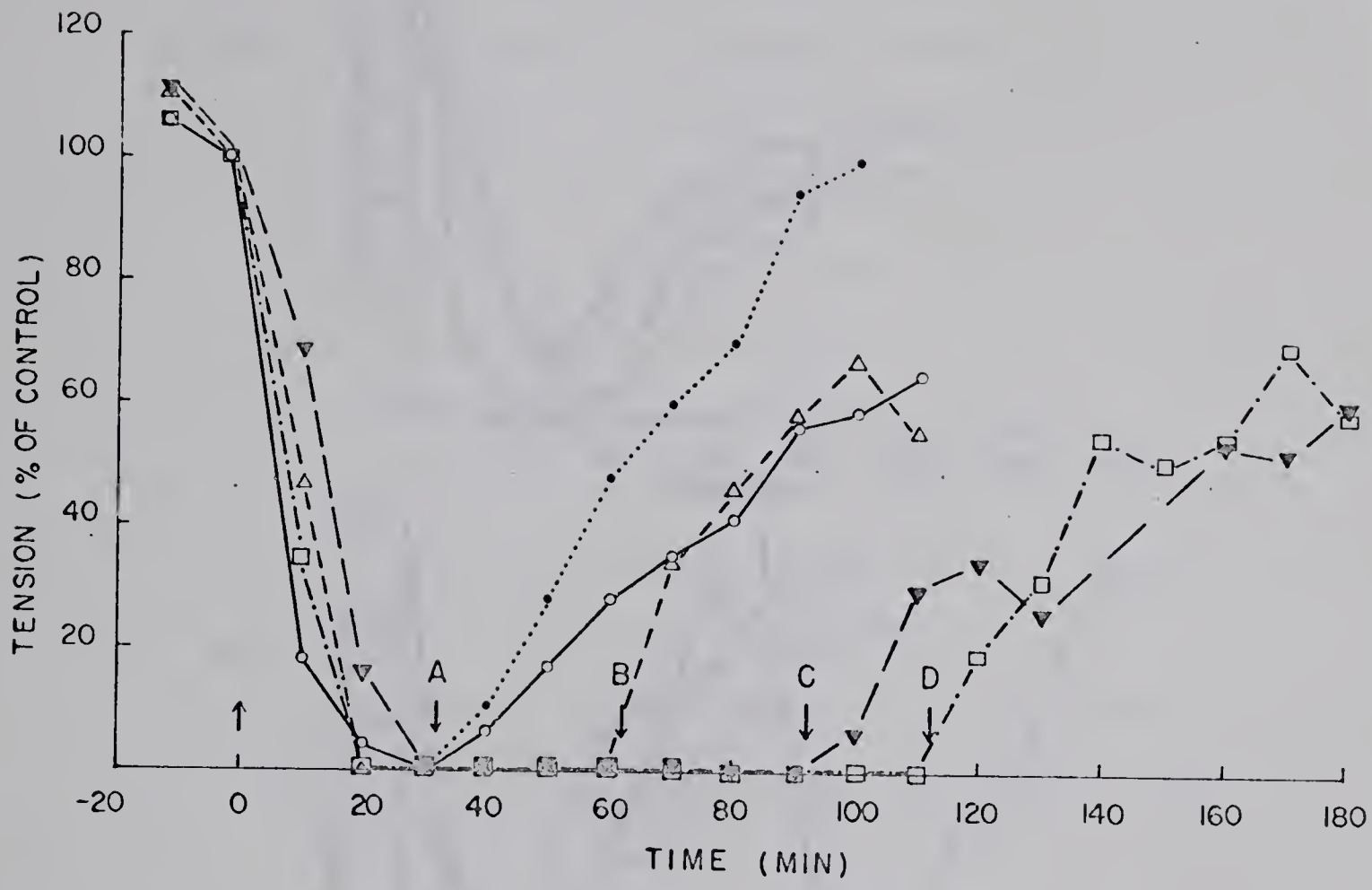

Fig. (7). Elimination of isotonic KCl-induced contractures of rat lumbricale muscles by exposure of the muscles to a Ca^{++}-free Krebs solution. ▲, muscles in Ca^{++}-free solution at time 0. ▼, muscles placed in Krebs with Ca^{++} (2mM); Curve A (○——○), after 30 minutes in a Ca^{++}-free Krebs; Curve B (△- - -△), after 60 minutes in a Ca^{++}-free Krebs; Curve C (▽— —▽), after 90 minutes in Ca^{++}-free Krebs; Curve D (□- - -□), after 110 minutes in Ca^{++}-free Krebs. Dotted line, data for Curve A plotted as % of maximum contracture tension at 100 minutes.

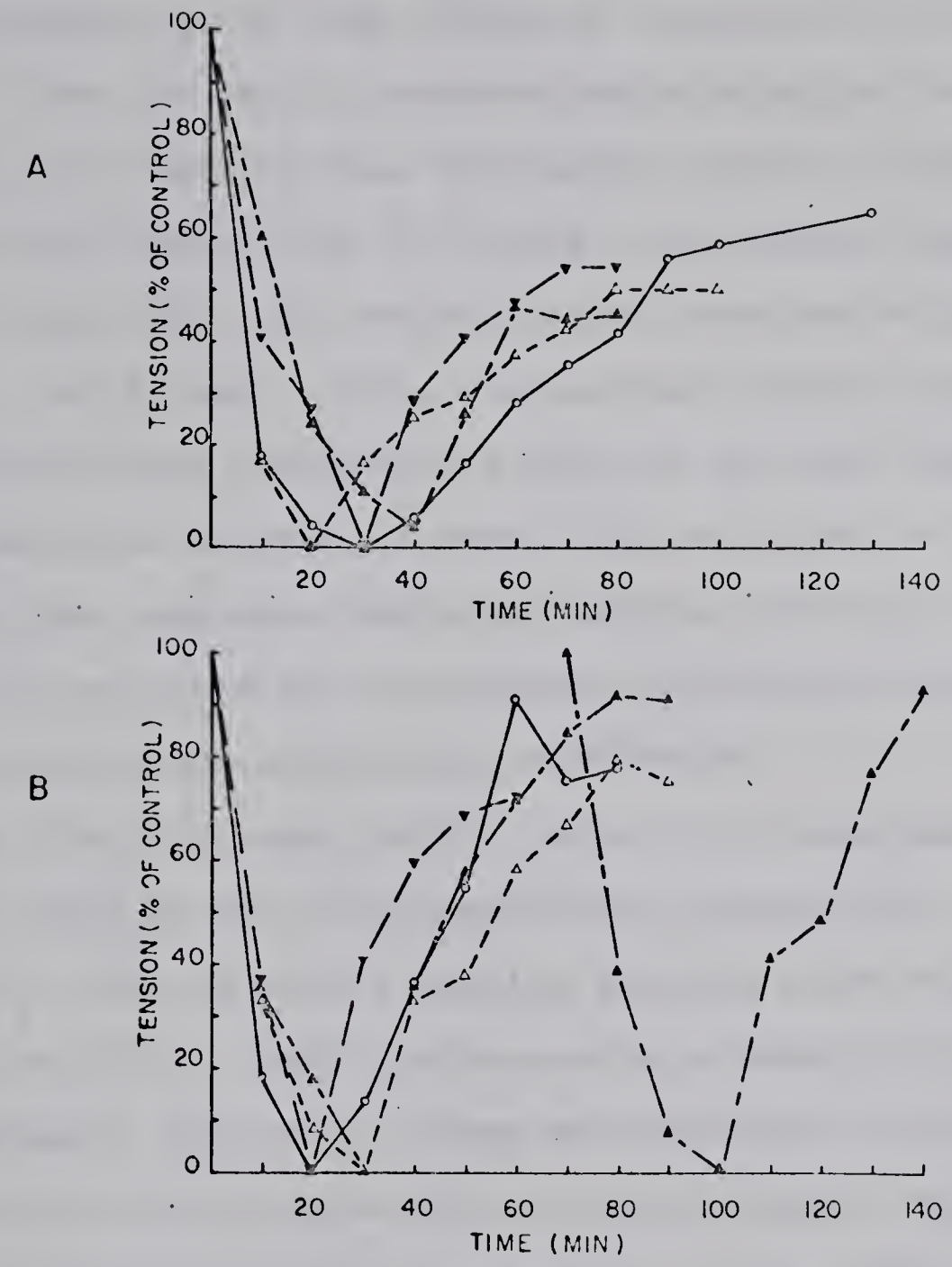

Fig. (8). Effect of a repeat test on the course of recovery of contracture tension of rat lumbricale muscles following reexposure to Krebs with Ca^{++}. A, initial exposure to Ca^{++}-free Krebs at time 0; B, second exposure of same muscles to Ca^{++}-free Krebs at time 0. A third exposure of one muscle to Ca^{++}-free Krebs at 70 minutes is shown at the far right of B. Five different muscles represented by different symbols. Muscles reexposed to Krebs with Ca^{++} one minute after the test with the smallest response recorded on each curve.

Figure 8 demonstrates the effects of repeated exposures to a Ca^{++}-free Krebs on the course of recovery of contracture tension. After an initial contracture elimination and recovery (A), the same muscles were again exposed to the Ca^{++}-free Krebs and then to the Ca^{++}Krebs to determine the recovery slopes (B). In one muscle the same procedure was repeated a third time. In B, the maximum tension height during the previous recovery was used as the 100% response. Not only were the maximum recovery tensions closer to the pre Ca^{++}-free Krebs exposure controls, but the recovery slopes also more clearly mirrored the contracture elimination slopes after the second and third contracture elimination.

In all the ion substitution tests to be described, lumbricales were used at 37°C with continuous oxygenation with 95%O_2-5%CO_2. The pH of all bathing solutions was adjusted to 7.4 ± 0.1 at 37°C. IsoKCl solutions were freshly prepared for each experiment, and in all cases were modified to match the divalent cation composition of the Krebs solution being used. In the figures that follow, the control contracture tensions (100% responses) were the values obtained just prior to the addition of a divalent cation other than Ca^{++} or to contracture elimination in a Ca^{++}-free Krebs solution.

III (b) Effects of excess Mg^{++} in place of Ca^{++} on isotonic KCl-induced contractures

The effects of excess Mg^{++} substitution for Ca^{++} on IsoKCl-induced contractures in rat lumbricale muscles are illustrated

in Figs. 9 and 10. In a total of 6 experiments, Mg^{++} in concentrations of 3.2mM (2mM above the 1.2mM present in normal Krebs) was not capable of supporting IsoKCl-induced contractures. In Fig. 9, the 3 most extensive experiments are plotted. In one experiment, the muscle was kept in the Ca^{++}-free, elevated Mg^{++} Krebs solution for 30 minutes instead of the usual 40 minutes (the arrow shows the introduction of Ca^{++}, elevated Mg^{++} Krebs). In this case there appeared to be less of a depressant effect due to the Mg^{++}.

In Fig. 10, typical contracture responses corresponding to sections A - D in Fig. 9 are shown. A shows a control response, B the lack of response in a Ca^{++}-free, elevated Mg^{++} Krebs. When Ca^{++}, elevated Mg^{++} was tested (C), there was possibly a small phasic response followed by a tonic response, or merely a tonic response. Maximum tensions were well below the control values. Following reduction of the Mg^{++} concentration (D), only low, tonic responses were generally obtained. While these responses had slightly greater maximum tensions than the responses in Ca^{++}, elevated Mg^{++} Krebs solution, these tensions were still below control values.

It appears from these results that exposure to elevated Mg^{++} had a lasting depressant effect which was at least partially dependent upon the length of time in the Ca^{++}-free, elevated Mg^{++} Krebs solution.

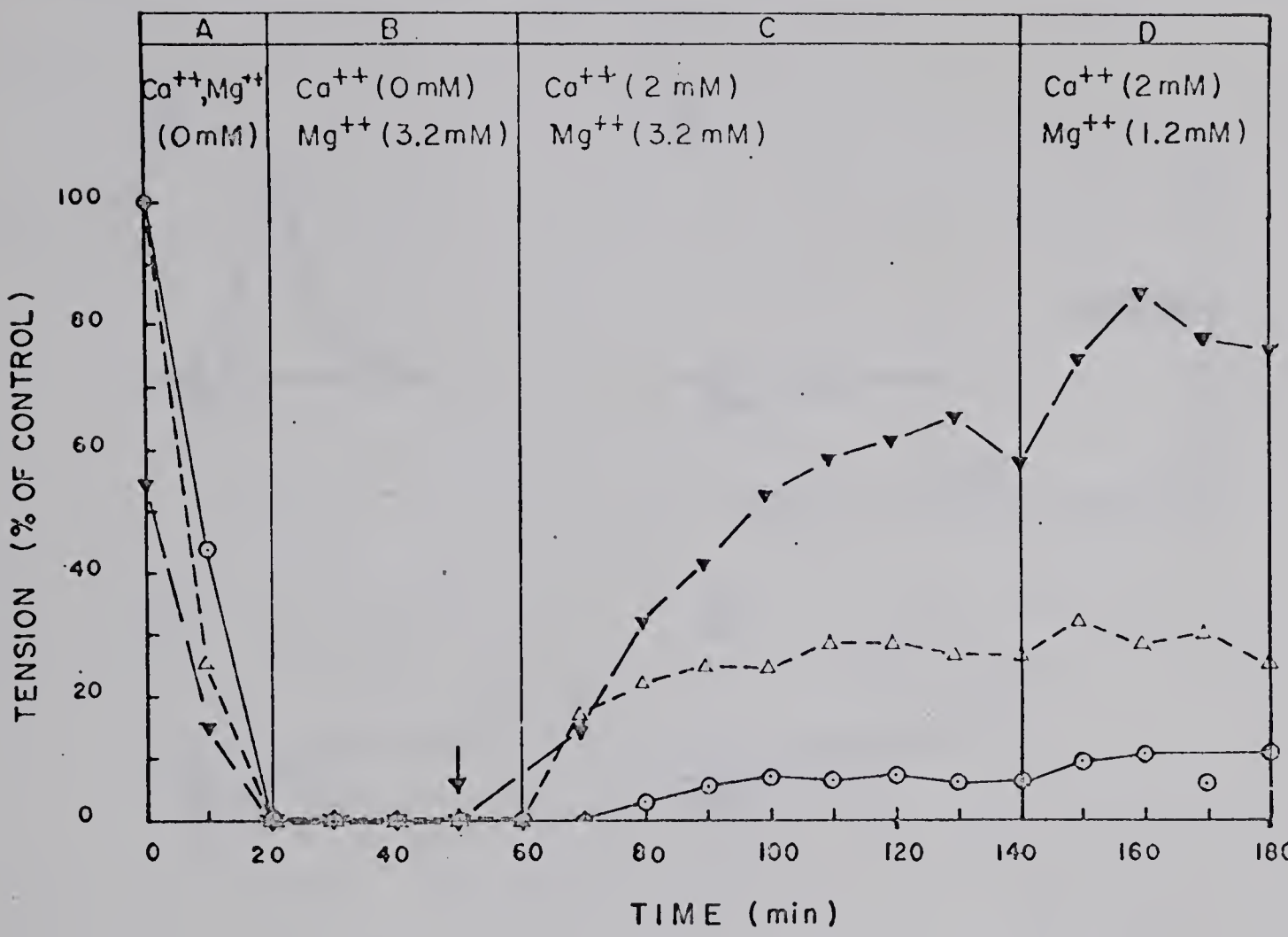

Fig. (9). Effects of substituting excess Mg^{++} for Ca^{++} on isotonic KCl-induced contractures in rat lumbricale muscles. A; IsoKCl contractures eliminated in Ca^{++}-free, Mg^{++}-free Krebs. B; IsoKCl contractures in Ca^{++}-free, elevated Mg^{++} Krebs. C; IsoKCl contractures in Ca^{++}, elevated Mg^{++} Krebs. D; IsoKCl contractures in normal Ca^{++}, Mg^{++} Krebs. ▽, introduction of Ca^{++}, elevated Mg^{++} Krebs in one curve (▽ —▽), kept in Ca^{++}-free, elevated Mg^{++} for 30 minutes instead of the usual 40 minutes. Results of 3 experiments are shown.

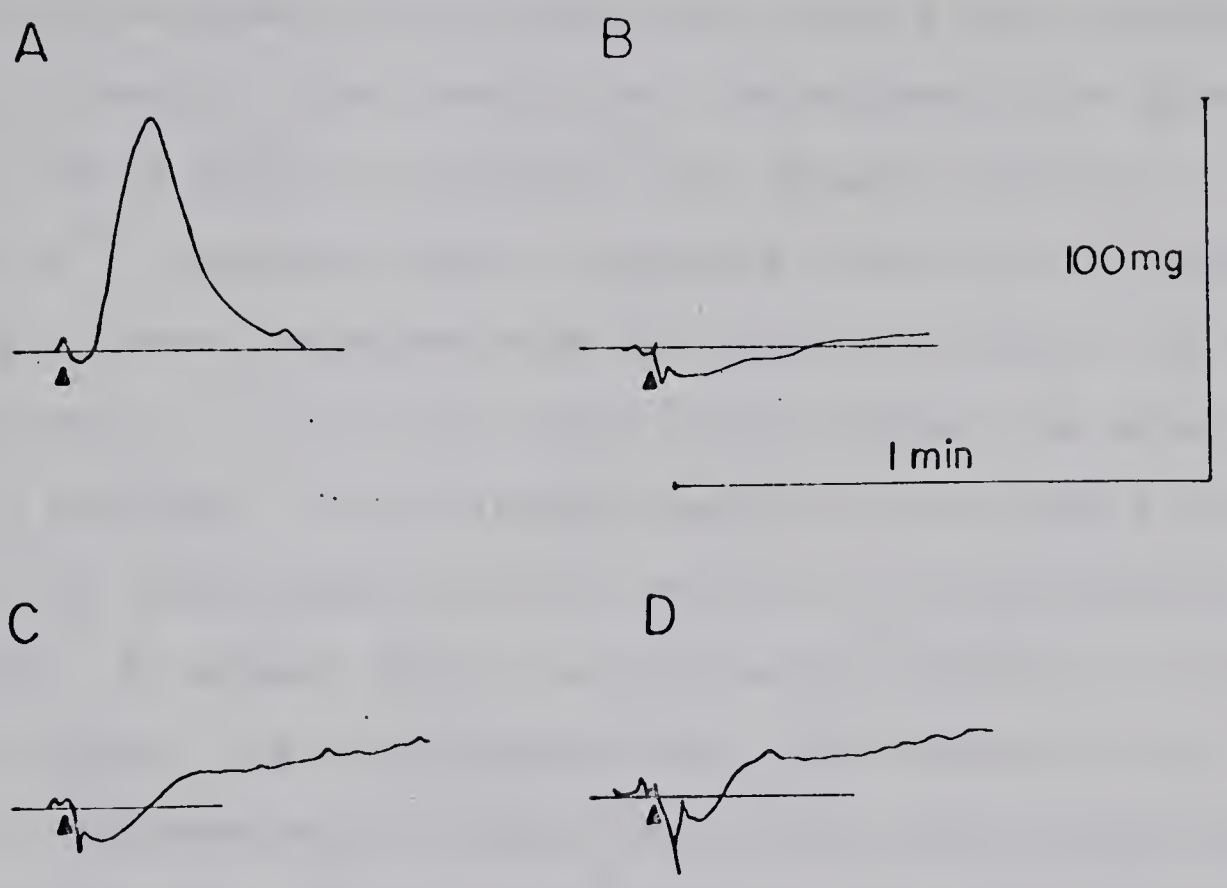

Fig. (10). Effects of substituting excess Mg^{++} for Ca^{++} on isotonic KCl-induced contractures in the rat lumbricale. A-D correspond to sections in Fig. 9. A; control IsoKCl response in normal Ca^{++} (2mM), Mg^{++} (1.2mM) Krebs. B; IsoKCl response after 20 minutes in Ca^{++}-free, elevated Mg^{++} (3.2mM) Krebs. C; IsoKCl response after 80 minutes in Ca^{++} (2mM), elevated Mg^{++} (3.2mM) Krebs. D; IsoKCl response after 20 minutes in normal Ca^{++} (2mM), Mg^{++} (1.2mM) Krebs.

III (c) Effects of Ba^{++} substitution for Ca^{++} on isotonic KCl-induced contractures

The effects of Ba^{++} substitution for Ca^{++} on IsoKCl-induced contractures in rat lumbricale muscles are illustrated in Figs. 11 and 12. The results of 3 experiments are shown in Fig. 11, one of which is complete only through section C.

The Ba^{++} supported IsoKCl responses were very difficult to typify. These responses were all tonic in nature, and were characterized by a lag of variable length before the onset of the tonic response. This variable period of lag before the onset of the tonic contracture increased as the testing in Ba^{++} progressed. In effect then, the contracture shifted to the right with time, and measurements made at a constant time after the introduction of IsoKCl showed decreased heights as testing progressed. The maximum tensions were difficult to determine because they either varied greatly in time of occurrence or never occurred, i.e. the tonic response merely kept increasing as long as the muscle was exposed to IsoKCl. Over a period of several minutes, these tonic responses could become quite large (e.g. 65-80% of control), and appeared capable of increasing further in some cases.

In one experiment shown in Fig. 11 (circles connected by a solid line), responses were measured in sections B - D at a constant time of 50 seconds after the administration of IsoKCl, or about 5 times the time required for the development of the maximum tension of Ca^{++} IsoKCl-induced contractures in this preparation. In the two remaining curves, maxima were measured

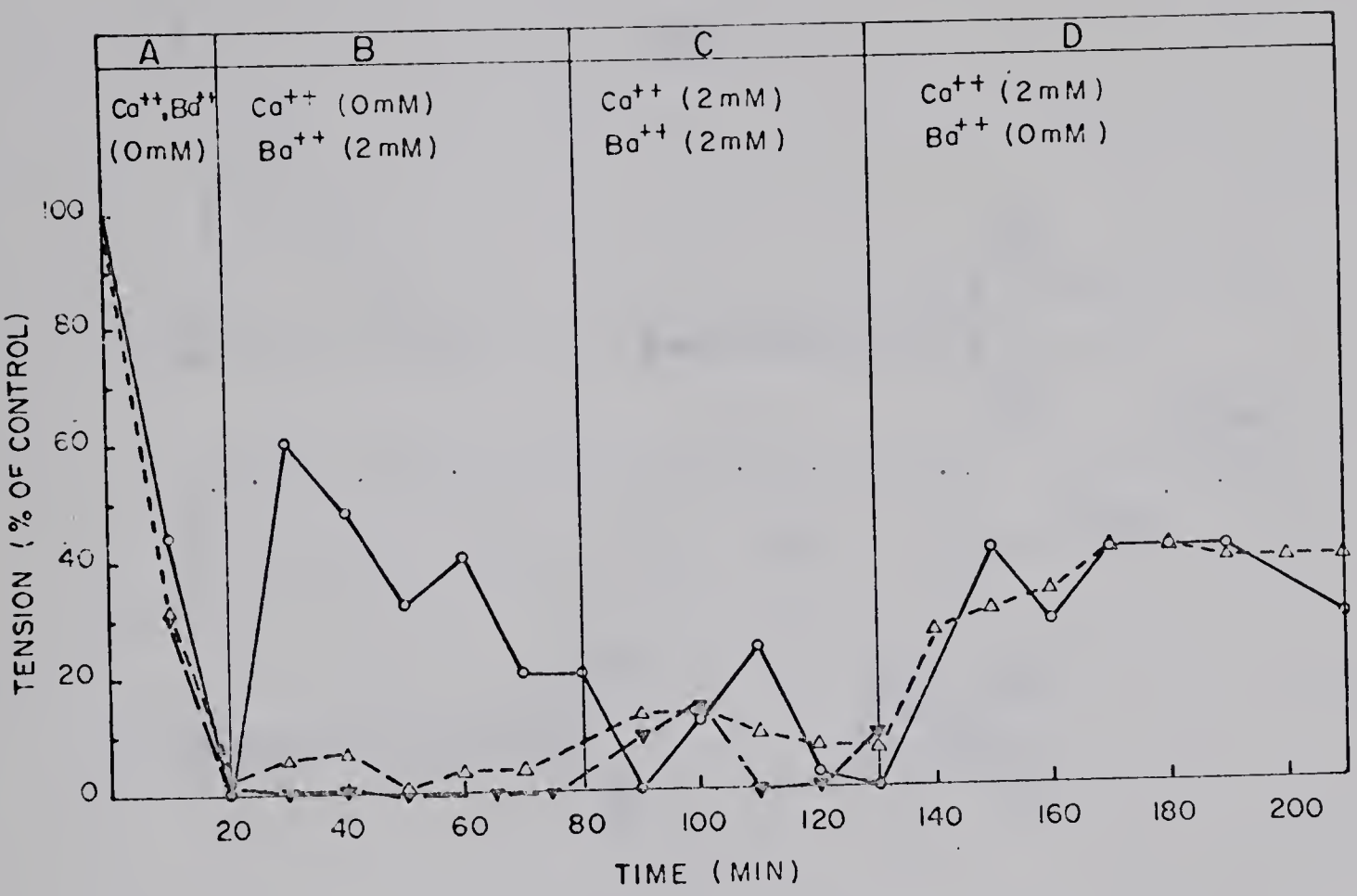

Fig. (11). Effects of substituting Ba^{++} for Ca^{++} on isotonic KCl-induced contractures in rat lumbricale muscles. A; IsoKCl contractures eliminated in a Ca^{++}-free Krebs. B; IsoKCl contractures in Ca^{++}-free, Ba^{++} Krebs. C; IsoKCl contractures in Ca^{++}, Ba^{++} Krebs. D; IsoKCl contractures in Ca^{++} Krebs. Results of 3 experiments are shown. (O——O), responses in sections B-D measured at constant time of 50 seconds after test solution administration. Responses of remaining two curves measured at maxima. One curve (▽——▽), is complete only through section C.

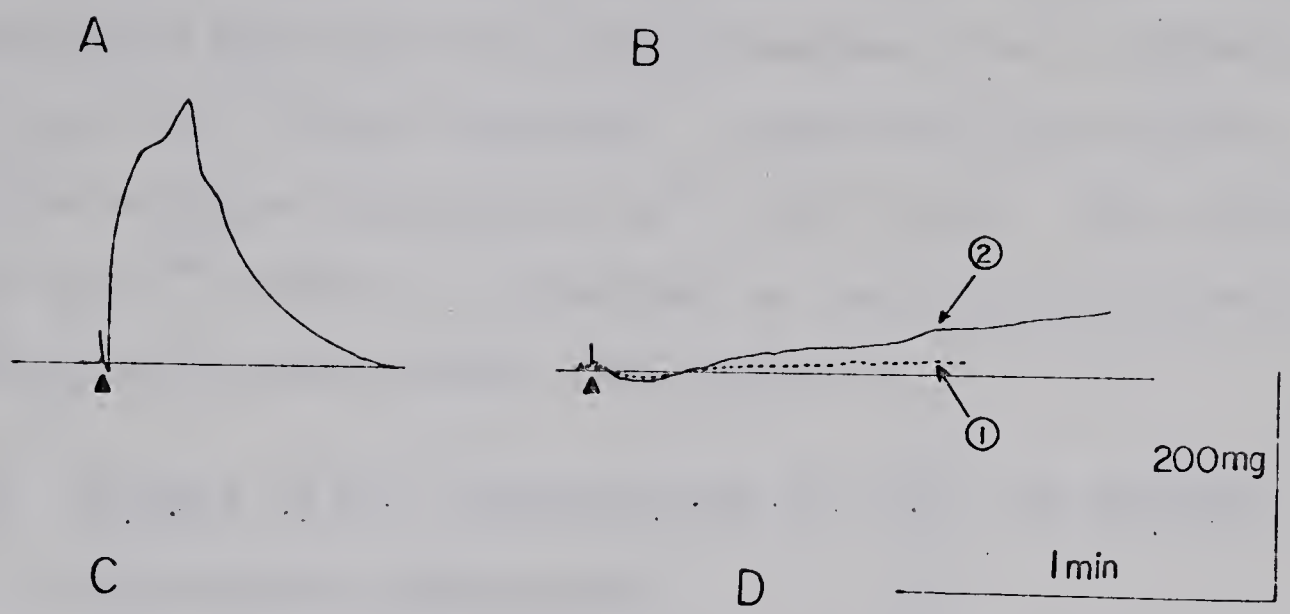

Fig. (12). Effects of substituting Ba^{++} for Ca^{++} on isotonic KCl-induced contractures in the rat lumbricale. A-D correspond to sections in Fig. 11. A; control IsoKCl response in Ca^{++} (2mM) Krebs. B; IsoKCl responses after (1) 10, and (2) 60 minutes in Ca^{++}-free, Ba^{++} (2mM) Krebs. C; IsoKCl responses after (1) 10, and (2) 40 minutes in Ca^{++} (2mM), Ba^{++} (2mM) Krebs. D; IsoKCl responses after (1) 10, and (2) 60 minutes in Ca^{++} (2mM) Krebs.

in every test, since these occurred within 1 or 2 minutes.

In Fig. 12, typical responses corresponding to sections A - D in Fig. 11 are shown. A shows a control response, while B demonstrates the variable, tonic responses that occurred in a Ca^{++}-free, Ba^{++} Krebs solution. C typifies the variable, tonic contractures obtained in Ca^{++}, Ba^{++} Krebs. The reintroduction of Ca^{++} Krebs in D resulted in the return of phasic responses which were smaller than control.

III (d) Effects of Ni^{++} substitution for Ca^{++} on isotonic KCl-induced contractures

The effects of Ni^{++} substitution for Ca^{++} on IsoKCl-induced contractures in rat lumbricale muscles are illustrated in Figs. 13, 14, and 15. The Ni^{++} supported contractures, like Ba^{++} responses, were difficult to typify. These responses were variable and characterized by tonic, slowly developing contractures of low amplitude. In all cases, however, attempts were made to measure maximum tensions.

Fig. 13 contains plots of the results obtained in 3 experiments. Fig. 14 shows typical responses corresponding to sections A - D in Fig. 13. A demonstrates a control IsoKCl response and B the tonic response in a Ca^{++}-free, Ni^{++} Krebs solution. A tonic response was also obtained in a Ca^{++}, Ni^{++} Krebs solution (C), although this response was of somewhat higher amplitude. Reintroduction of Ca^{++} Krebs resulted in potentiated, phasic responses that were somewhat erratic in shape (D).

Fig. 15 demonstrates the effects of a Ca^{++}, Ni^{++} Krebs

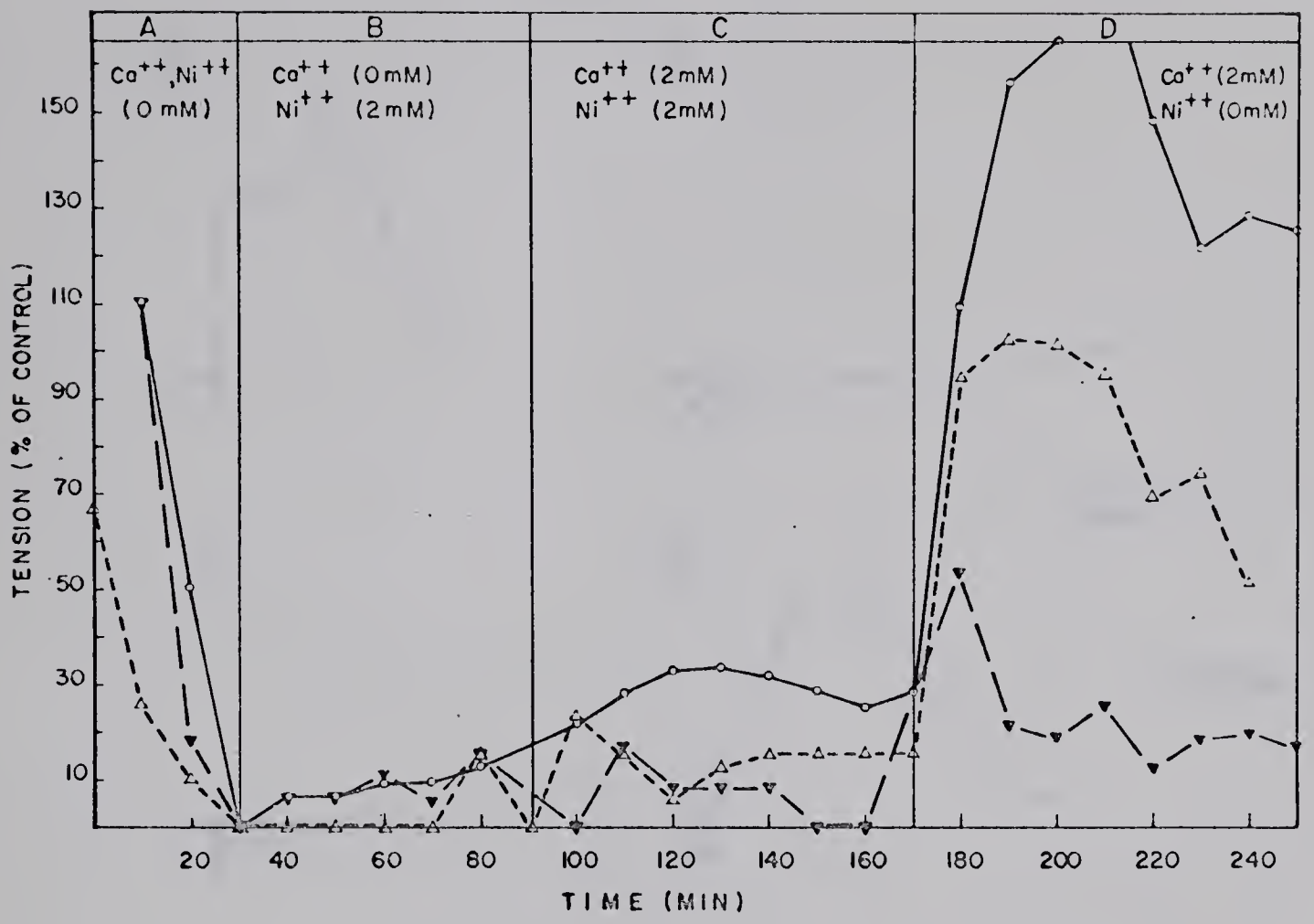

Fig. (13). Effects of substituting Ni^{++} for Ca^{++} on isotonic KCl-induced contractures in rat lumbricale muscles. A; IsoKCl contractures eliminated in a Ca^{++}-free Krebs. B; IsoKCl contractures in Ca^{++}-free, Ni^{++} Krebs. C; IsoKCl contractures in Ca^{++}, Ni^{++} Krebs. D; IsoKCl contractures in Ca^{++} Krebs. Results of 3 experiments are shown.

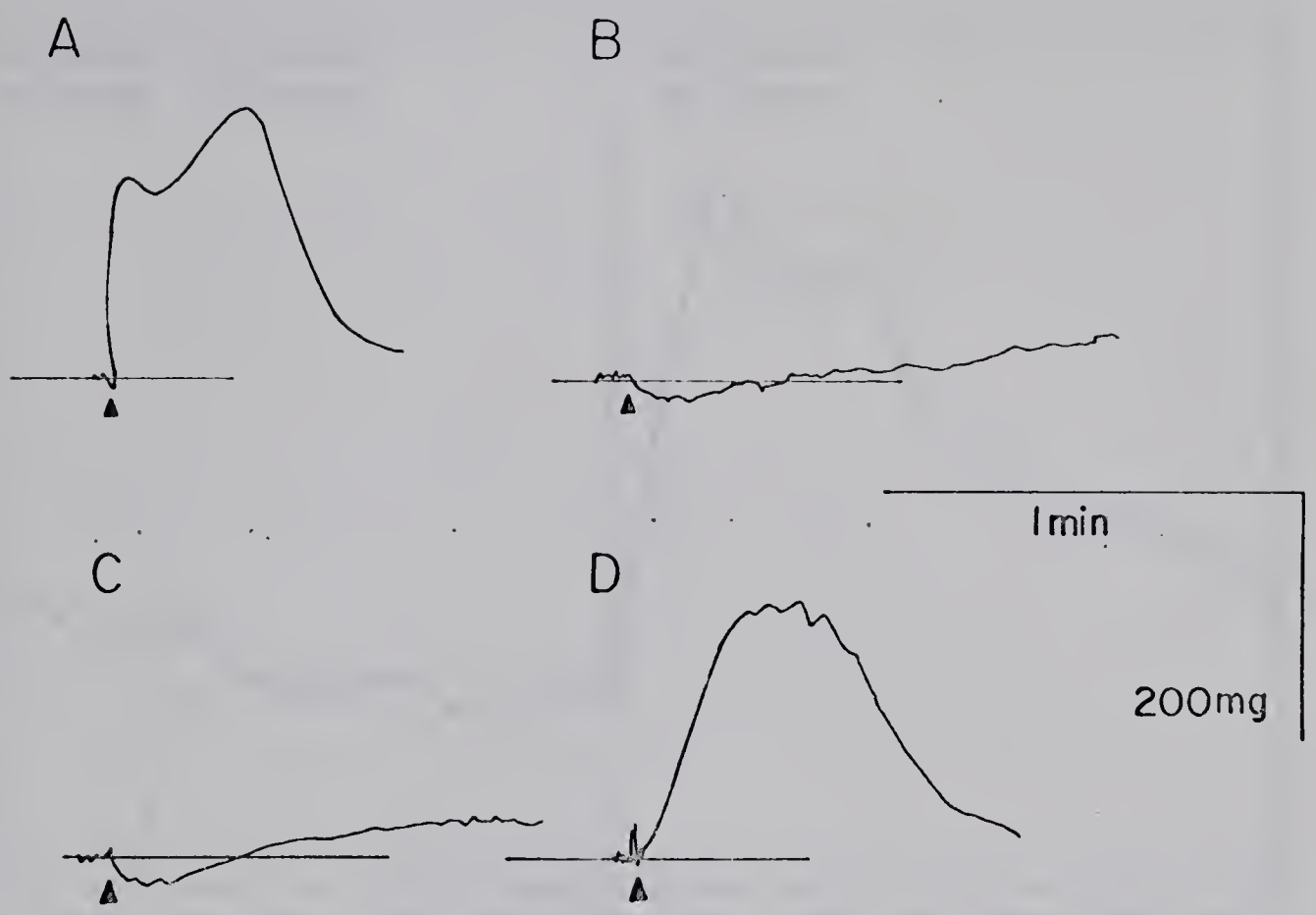

Fig. (14). Effects of substituting Ni^{++} for Ca^{++} on isotonic KCl-induced contractures in the rat lumbricale. A-D correspond to sections in Fig. 13. A; control IsoKCl response in Ca^{++} (2mM) Krebs. B; IsoKCl response after 50 minutes in Ca^{++}-free, Ni^{++} (2mM) Krebs. C; IsoKCl response after 65 minutes in Ca^{++} (2mM), Ni^{++} (2mM) Krebs. D; IsoKCl response after 10 minutes in Ca^{++} (2mM) Krebs.

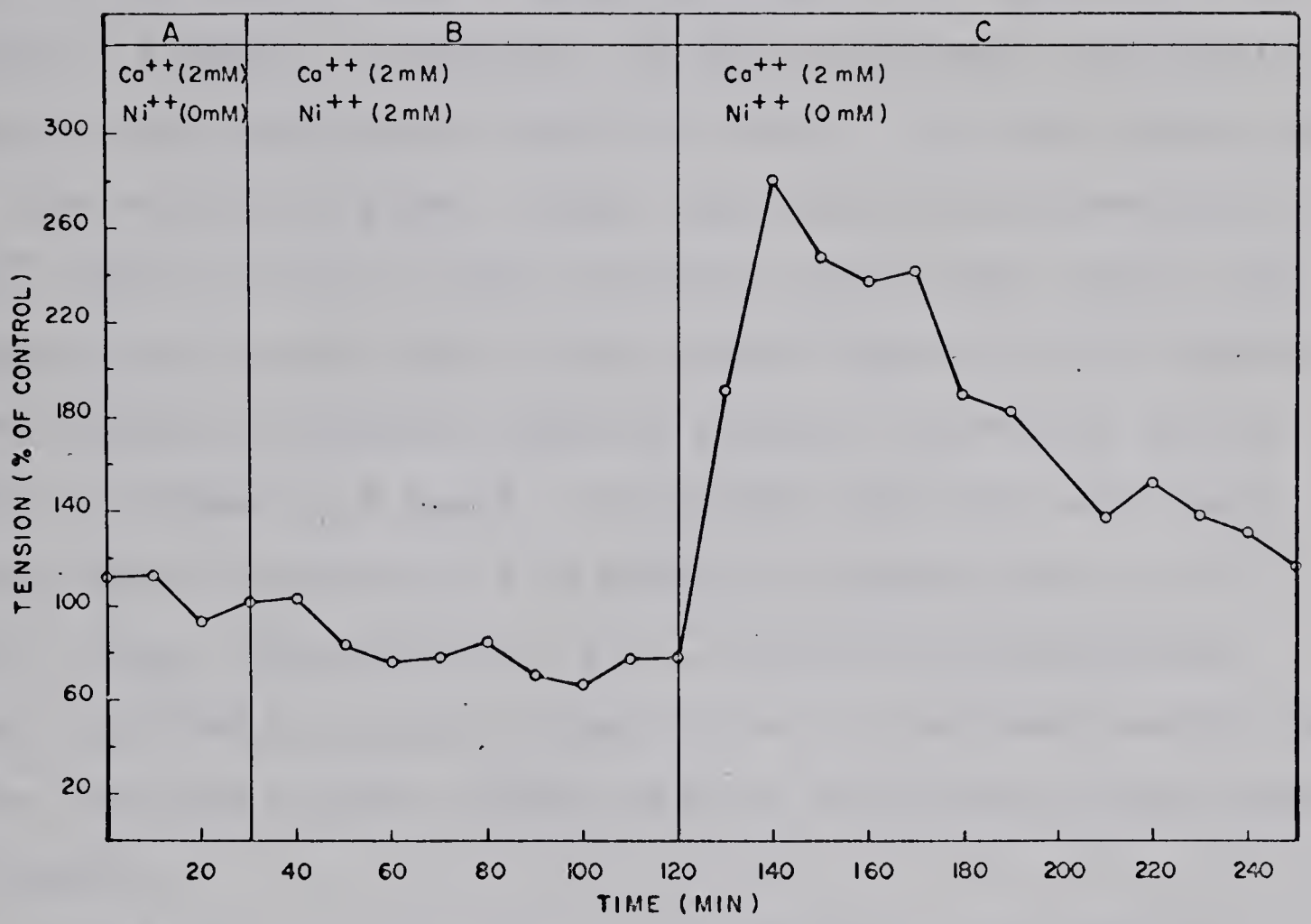

Fig. (15). Effects of Ni^{++} and Ca^{++} on isotonic KCl-induced contractures in the rat lumbricale. A; control IsoKCl contractures in Ca^{++} Krebs. B; IsoKCl contractures in Ca^{++}, Ni^{++} Krebs. C; IsoKCl contractures in Ca^{++} Krebs.

solution on the maximum tensions of IsoKCl contractures when the muscle was exposed to this solution directly after Ca^{++} Krebs. The contracture tensions remained near 80% of control after 1.5 hours of testing. In this experiment, the initial part of the contractures remained phasic, but they demonstrated a slow relaxation phase. After the return of the muscle to a Ca^{++} Krebs solution, IsoKCl produced potentiated, phasic responses that reached 280% of the control tension in 20 minutes. The maximum contracture tensions then fell gradually to near control values in 2 hours. In another identical experiment, contracture tensions fell in height to become tonic in Ca^{++}, Ni^{++} Krebs. Subsequently, in Ca^{++} Krebs the contractures were typified by a rapid, phasic rise to a maximum tension, but they remained at this tension without relaxation in the presence of IsoKCl.

In 2 other experiments, the preparation was placed in a Ca^{++}-free, Ni^{++} Krebs directly after Ca^{++} Krebs. In these experiments the contractures underwent a transition from a phasic response to a slowly developing tonic response. In one experiment these tonic responses stabilized after decreasing in amplitude, while in the other they decreased and then gradually increased. Return to a Ca^{++} Krebs led to the return of phasic responses which were potentiated in both cases to approximately 150% of the initial control.

III (e) Effects of Co^{++} substitution for Ca^{++} on isotonic KCl-induced contractures

The effects of Co^{++} substitution for Ca^{++} on IsoKCl-induced contractures in rat lumbricale muscles are illustrated in Figs. 16, 17, and 18.

In Fig. 16 are plotted the results obtained in 3 experiments. Fig. 17 shows typical responses corresponding to the experimental conditions in sections A - D of Fig. 16. The control IsoKCl response is shown in Fig. 17-A. Transient, phasic contractures were obtained in a Ca^{++}-free, Co^{++} Krebs as shown in Fig. 17-B-1. These contractures were found to become less and less phasic with time in this solution until only tonic responses were obtained (Fig. 17-B-2). In a Ca^{++}, Co^{++} Krebs solution (Fig. 17-C), larger contractures were obtained with a rapid contracture onset, but after attaining the maxima this tension was maintained; there was no clear relaxation phase in IsoKCl. The slope of the contracture recovery curve in Ca^{++}, Co^{++} Krebs solution closely resembled that seen with Ca^{++} after contracture elimination in a Ca^{++}-free medium (see Figs. 7 and 8). The maximum tension in Ca^{++}, Co^{++} Krebs, however, was higher than the 60-70% seen in control Ca^{++} recovery. Upon removal of the Co^{++} (Fig. 17-D), potentiated, phasic contractures were obtained. In one of the experiments shown in Fig. 16, Co^{++} did not support IsoKCl contractures in the absence of Ca^{++}.

Fig. 18 demonstrates the effects of a Ca^{++}, Co^{++} Krebs solution introduced directly after Ca^{++} Krebs. After an

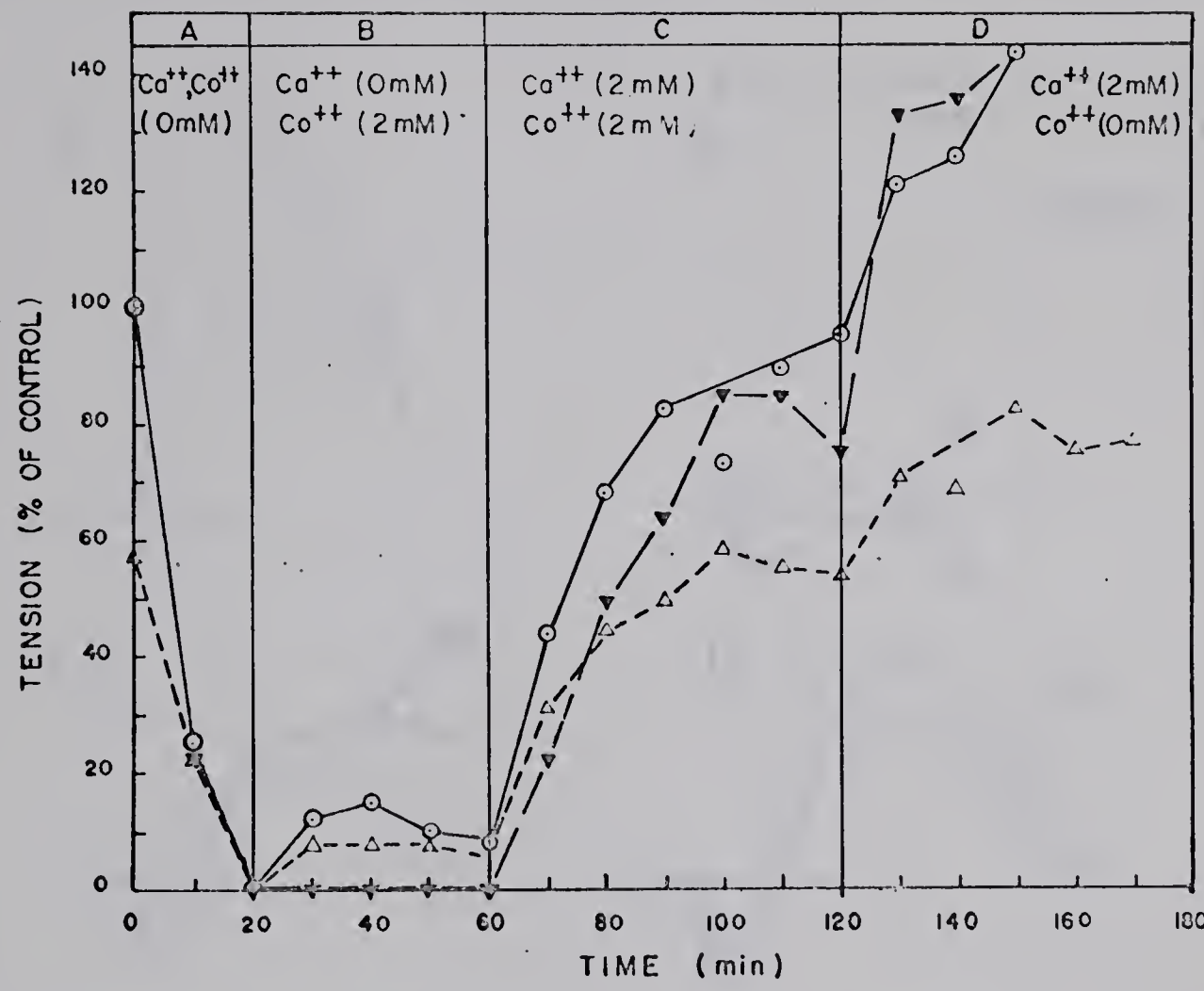

Fig. (16). Effects of substituting Co^{++} for Ca^{++} on isotonic KCl-induced contractures in rat lumbricale muscles. A; IsoKCl contractures eliminated in Ca^{++}-free Krebs. B; IsoKCl contractures in Ca^{++}-free, Co^{++} Krebs. C; IsoKCl contractures in Ca^{++}, Co^{++} Krebs. D; IsoKCl contractures in Ca^{++} Krebs. Results of 3 experiments are shown. One curve (▽—▽) did not show Co^{++} supported contractures.

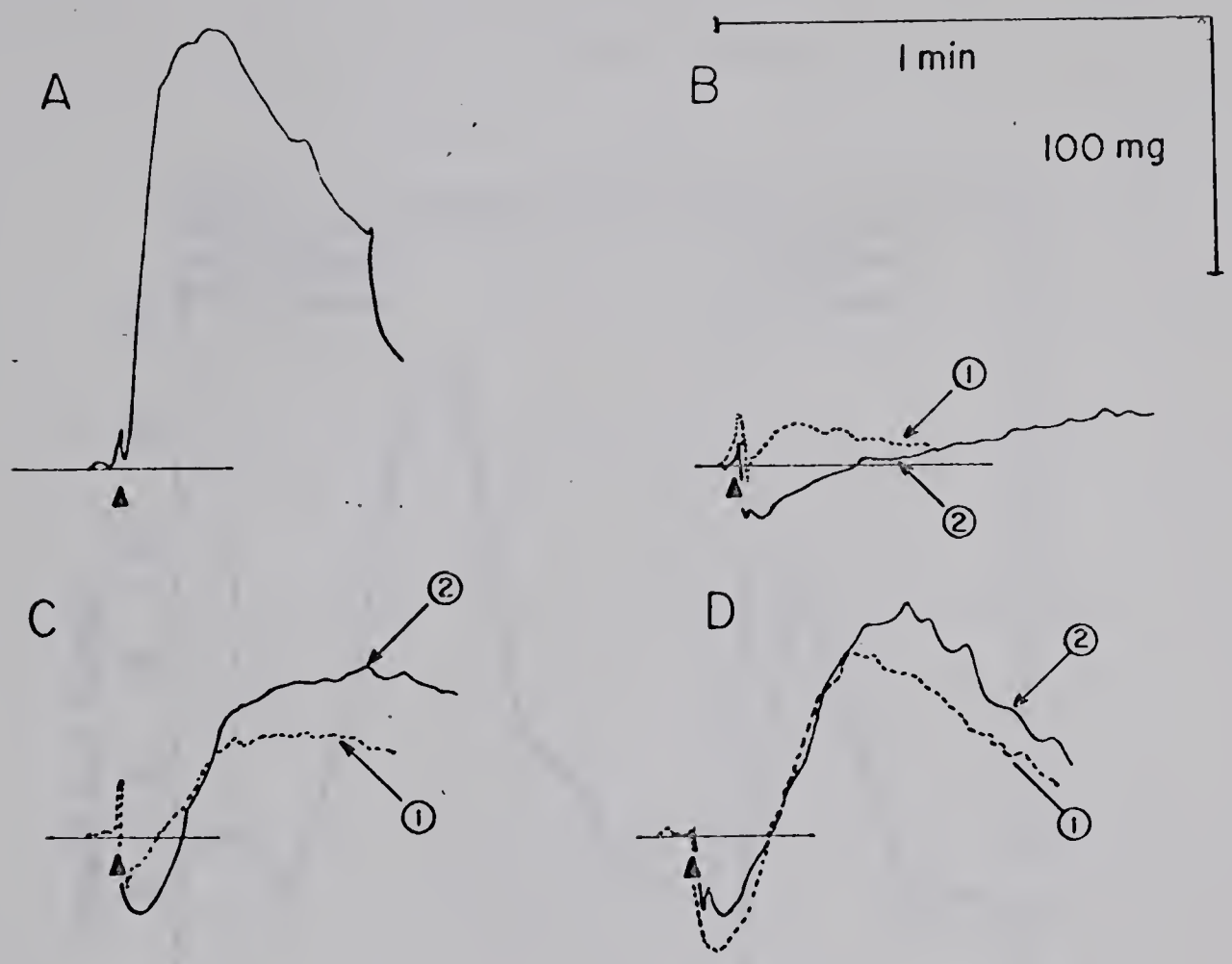

Fig. (17). Effects of substituting Co^{++} for Ca^{++} on isotonic KCl-induced contractures in the rat lumbricale. A-D correspond to sections in Fig. 16. A; control IsoKCl response in Ca^{++} (2mM) Krebs. B; IsoKCl responses after (1) 20, and (2) 50 minutes in Ca^{++}-free, Co^{++} (2mM) Krebs. C; IscKCl responses after (1) 10, and (2) 40 minutes in Ca^{++} (2mM), Co^{++} (2mM) Krebs. D; IsoKCl responses after (1) 10, and (2) 40 minutes in Ca^{++} (2mM) Krebs.

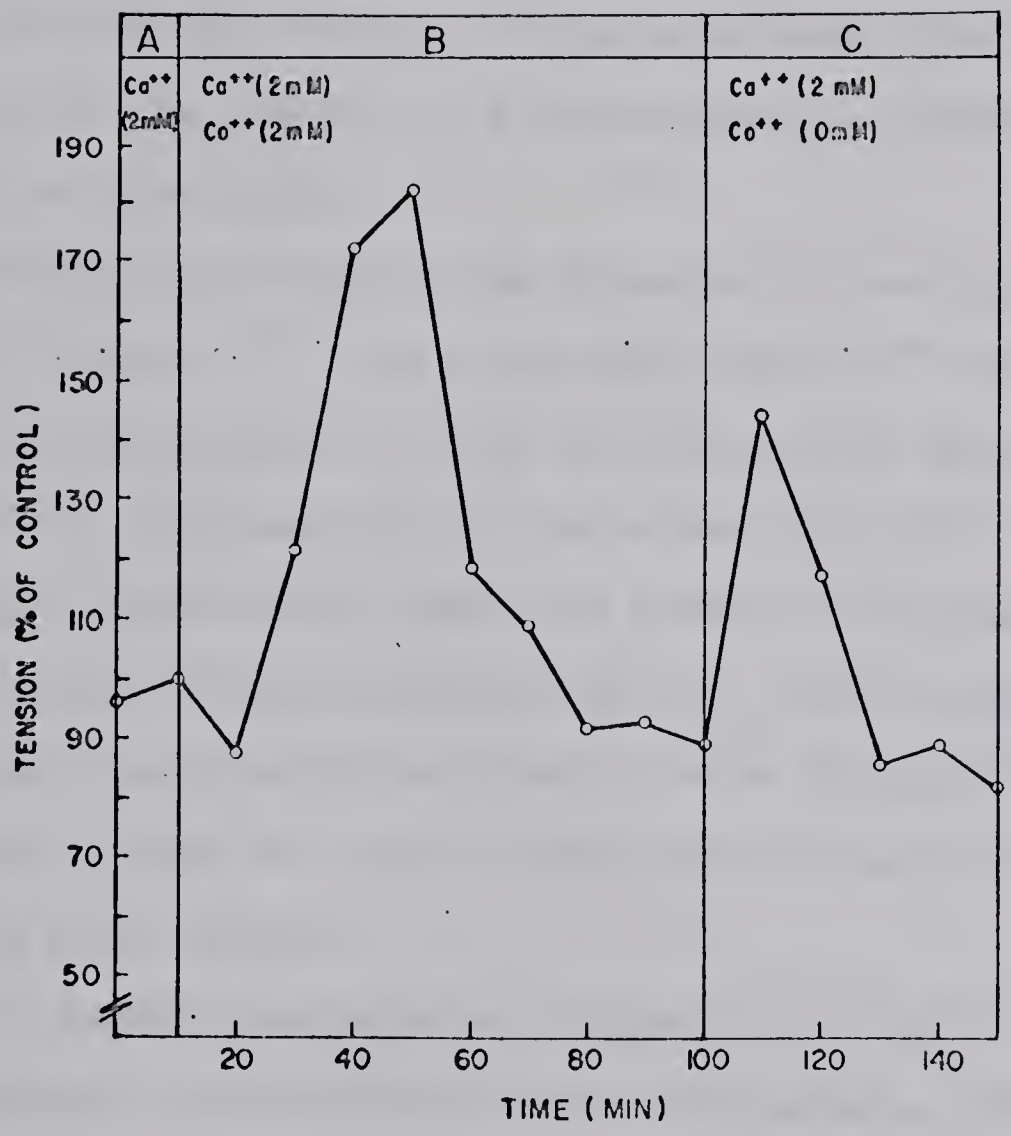

Fig. (18). Effects of Co^{++} and Ca^{++} on isotonic KCl-induced contractures in the rat lumbricale. A; control IsoKCl contractures in Ca^{++} Krebs. B; IsoKCl contractures in Ca^{++}, Co^{++} Krebs. C; IsoKCl contractures in Ca^{++} Krebs.

initial drop of contracture tension, the responses climbed to 182% of control and then decreased until stabilizing at 90% of the control tension. When placed in Ca^{++} Krebs again, the contracture tension again increased above control, and then fell to 80-85% of the control tension. These contractures were phasic, but over the course of the experiment they tended to become erratic in shape with a progressively increasing delay of the relaxation phase.

In a further experiment, the preparation was placed directly into a Ca^{++}-free, Co^{++} Krebs solution from Ca^{++} Krebs. After an initial potentiation to 115% of the control maximum tension in 20 minutes, the contracture responses fell to 0 tension in 1 hour. These contractures remained phasic during exposure to Co^{++}. Upon returning the muscle to Ca^{++} Krebs solution, phasic contractures reaching 60% of control were obtained, but these contractures became erratic in shape with time and developed a secondary tonic phase.

Phasic IsoKCl contractures supported by Co^{++} (Fig. 17-B-1) were completely supplanted by tonic contractures (Fig. 17-B-2) within 5 IsoKCl tests (50 minutes in Co^{++} Krebs solution). A subsequent 20 mM caffeine test was strongly phasic, but had a slow relaxation phase. The reintroduction of Ca^{++} showed potentiated, erratic IsoKCl responses, and a phasic 20mM caffeine response that was larger than the first caffeine response in Co^{++}, but this caffeine response also relaxed slowly.

III (f) Effects of Sr^{++} substitution for Ca^{++} on isotonic KCl-induced contractures

The effects of Sr^{++} substitution for Ca^{++} on IsoKCl-induced contractures in rat lumbricale muscles are shown in Figs. 19, 20, and 21.

In Fig. 19 the results of 3 experiments are plotted. Fig. 20 shows typical responses corresponding to sections A - D in Fig. 19. In Fig. 20-A a control IsoKCl response is shown. Sr^{++} supported IsoKCl-induced responses in a Ca^{++}-free, Sr^{++} Krebs solution are shown in Fig. 20-B. When these contractures were superimposed, they appeared as a family of curves growing in height with time in a very regular fashion. When a Ca^{++}, Sr^{++} Krebs solution was introduced (Fig. 20-C), these contractures decreased in maximum tensions developed. These contractures became less and less sharply phasic with an increasing duration of exposure to the Ca^{++}, Sr^{++} Krebs solution until they approached the low, rounded contractures seen when the Sr^{++} was removed from the solution bathing the muscle (Fig. 20-D).

In some cases, IsoKCl-induced responses in the presence of Sr^{++} without Ca^{++} did not increase in successive IsoKCl tests in a regular fashion, and demonstrated an initial phasic response followed by a secondary, slower relaxation phase. One curve (inverted, darkened triangles) is unusual in that a potentiation occurs upon transfer from Ca^{++}-free, Sr^{++} Krebs to Ca^{++}, Sr^{++} Krebs solution. In all 3 experiments the slopes of the contracture recovery curves are steeper than those seen with Ca^{++} after contracture elimination in a Ca^{++}-free medium

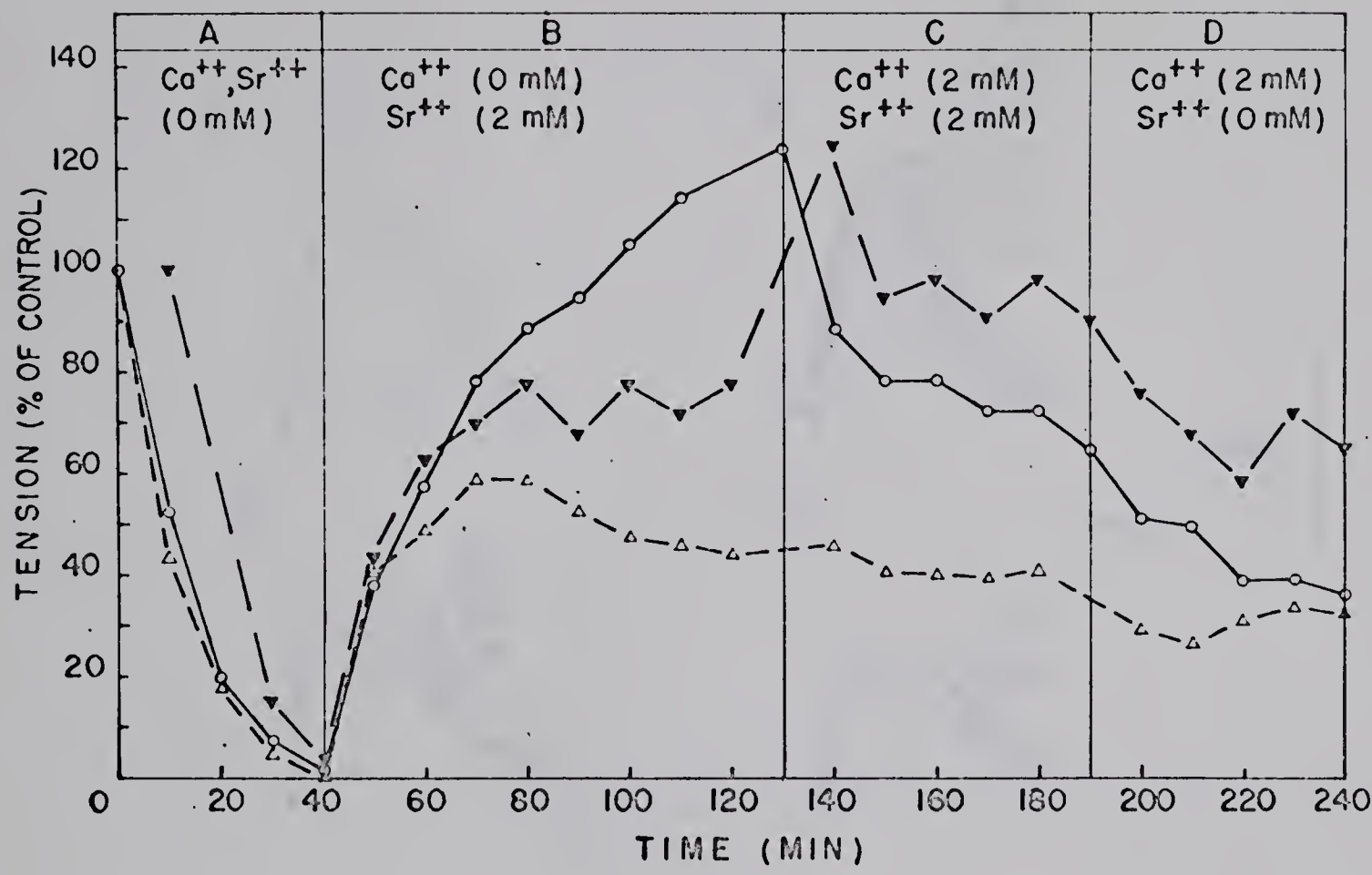

Fig. (19). Effects of substituting Sr^{++} for Ca^{++} on isotonic KCl-induced contractures in rat lumbricale muscles. A; IsoKCl contractures eliminated in Ca^{++}-free Krebs. B; IsoKCl contractures in Ca^{++}-free, Sr^{++} Krebs. C; IsoKCl contractures in Ca^{++}, Sr^{++} Krebs. D; IsoKCl contractures in Ca^{++} Krebs. Results of 3 experiments are shown.

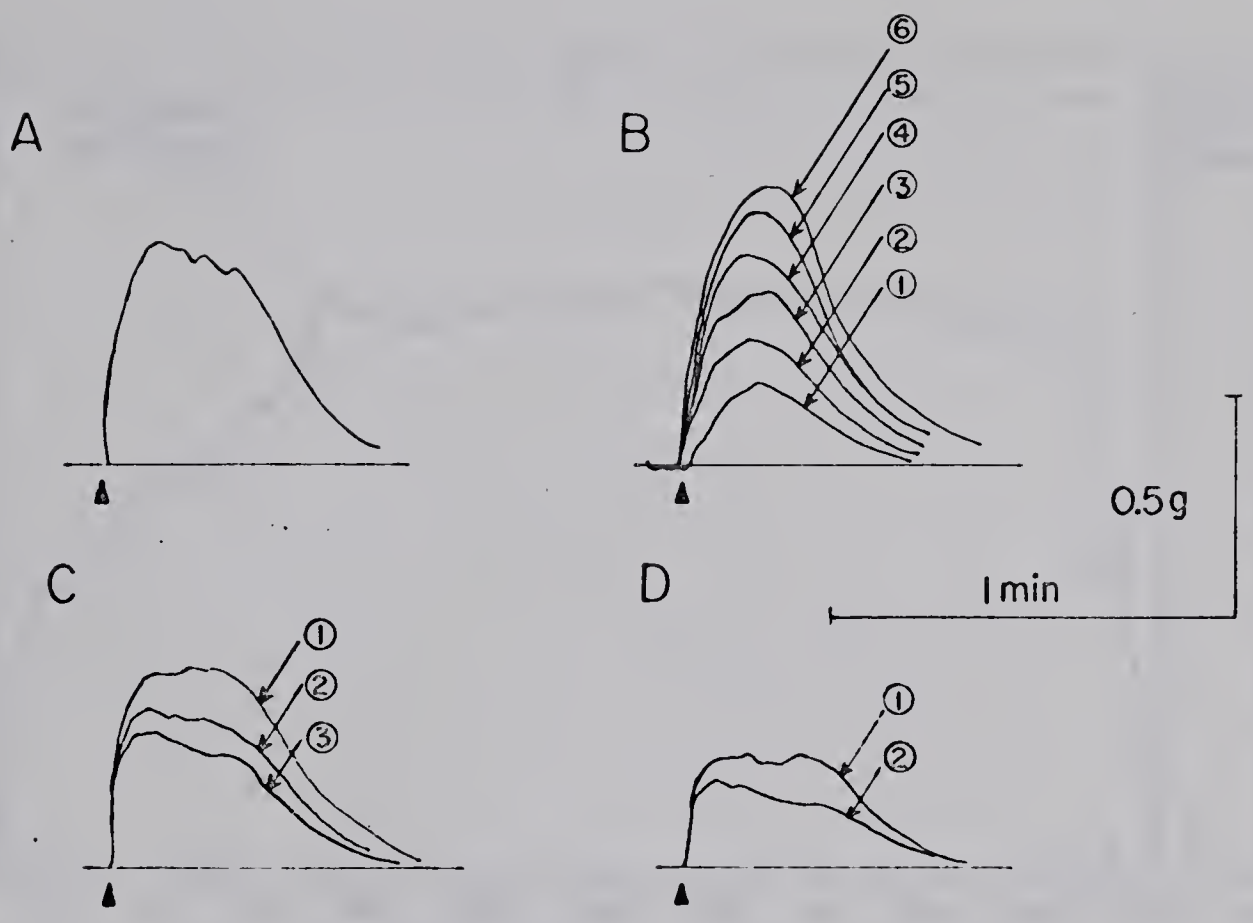

Fig. (20). Effects of Sr^{++} substitution on isotonic KCl-induced contractures in the rat lumbricale. A-D correspond to sections in Fig. 19. A; control IsoKCl response in Ca^{++} (2mM) Krebs. B; IsoKCl responses after (1) 10, (2) 20, (3) 30, (4) 50, (5) 70, and (6) 90 minutes in Ca^{++}-free, Sr^{++} (2mM) Krebs. C; IsoKCl responses after (1) 10, (2) 40, and (3) 80 minutes in Ca^{++} (2mM), Sr^{++} (2mM) Krebs. D; IsoKCl responses after (1) 10, and (2) 30 minutes in Ca^{++} (2mM) Krebs.

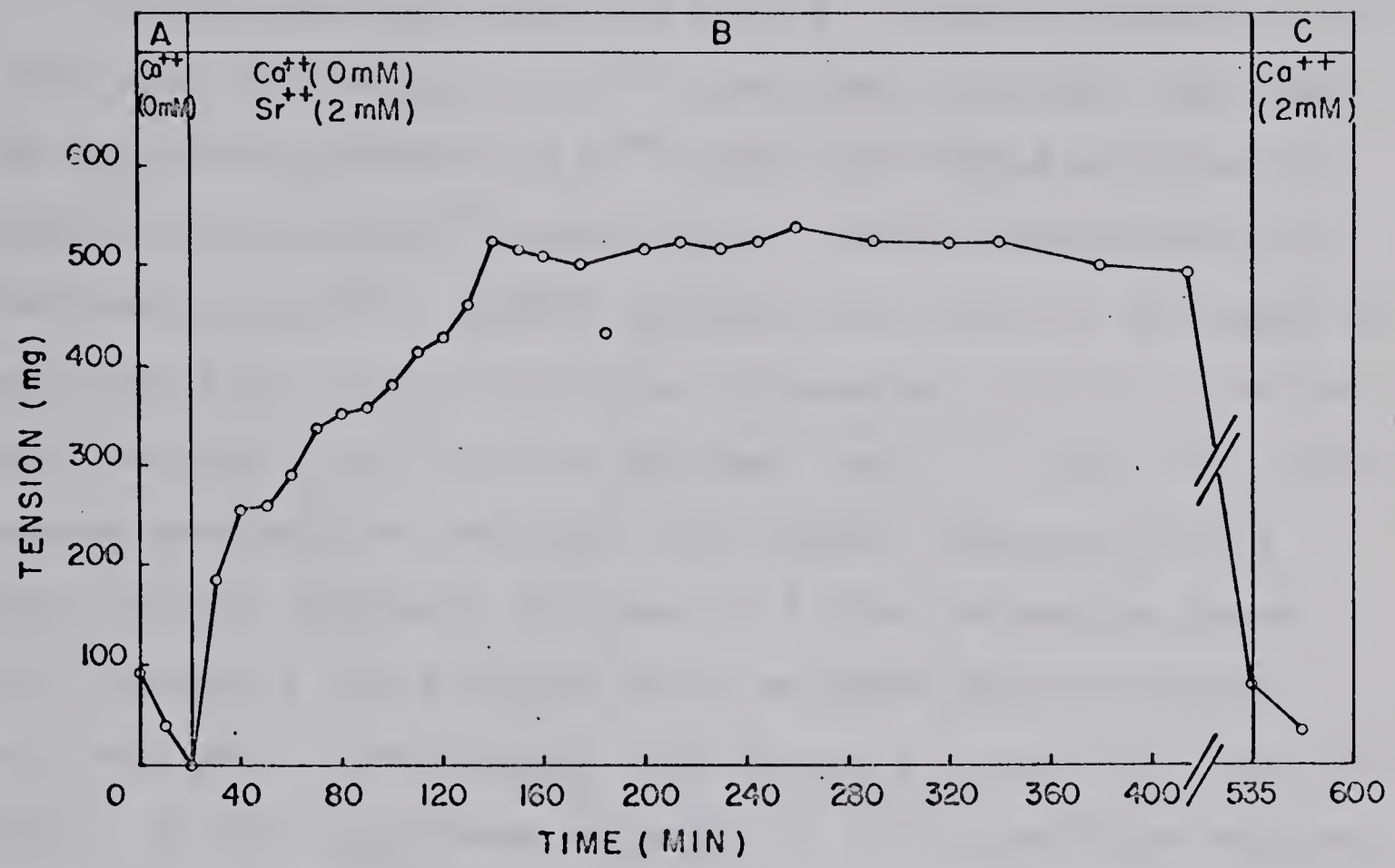

Fig. (21). Effects of Sr^{++} substitution on isotonic KCl-induced contractures in the rat lumbricale. A; IsoKCl contractures eliminated in Ca^{++}-free Krebs. B; IsoKCl contractures in Ca^{++}-free, Sr^{++} Krebs. Slash on the time axis represents a 2 hour lapse. C; IsoKCl contractures in Ca^{++} Krebs.

(see Figs. 7 and 8). The maximum tension in Ca^{++}, Sr^{++} Krebs in 2 of the 3 experiments is larger than the 60-70% seen in control Ca^{++} recovery.

In the experiment shown in Fig. 21, IsoKCl-induced contractures were eliminated in a Ca^{++}-free Krebs solution, and then the muscle was placed in a Ca^{++}-free, Sr^{++} Krebs solution to determine how long Sr^{++} would support IsoKCl-contractures in the absence of Ca^{++}. IsoKCl contractures steadily increased in amplitude for 2 hours and became increasingly phasic in nature. Over the next 4 hours and 40 minutes, the Sr^{++} supported IsoKCl-induced contractures remained very stable, demonstrating a sharp, phasic component followed by a slow relaxation phase. After another 2 hours during which no tests were performed, only very small contractures were obtained in both Sr^{++} and Ca^{++} Krebs. In this experiment attempts to obtain caffeine contractures in Sr^{++} and Ca^{++} were unsuccessful after time equals 535 minutes in Fig. 21-C.

In another experiment, the muscle was transferred directly from a Ca^{++} Krebs to a Ca^{++}, Sr^{++} Krebs solution. The maximum tension of IsoKCl-induced contractures rose rapidly to 193% of control within 30 minutes in the latter solution. These responses were phasic and remained at 182% of control after 1.5 hours in the Ca^{++}, Sr^{++} Krebs solution. Upon returning the muscle to a Ca^{++} Krebs solution, the tension of IsoKCl responses fell to control values within 20 minutes and declined gradually to 70% of control in 2 hours. In a further experiment, the preparation was transferred from a Ca^{++} Krebs to a Ca^{++}-free,

Sr^{++}Krebs solution. After an initial fall in contracture tension to 80% of control in the first 20 minutes, the contracture tensions rose to control values by 1 hour, and slightly above control after 2 hours in the Ca^{++}-free, Sr^{++} Krebs solution. Returning the muscle to a Ca^{++} Krebs solution resulted in a steady decline of contracture tension to about 30% of control by 1 hour. The contracture tensions stabilized shortly thereafter at 20% of control values.

III (g) Isotonic KCl- and caffeine-induced contractures supported by Sr^{++} after Ca^{++} depletion by caffeine.

As previously mentioned, in a Ca^{++}-free solution IsoKCl-induced contractures were usually eliminated after 20 minutes and only occasionally after 40-50 minutes exposure to the Ca^{++}-free solution. Considerably longer exposures to a Ca^{++}-free solution were required for elimination of caffeine contractures (e.g. 130 minutes in the experiment plotted in Fig. 22). Presumably, as previously shown for frog muscle (Frank, 1962), in rat skeletal muscle caffeine can utilize 'bound' stores of Ca^{++} in producing contractures. As shown in Fig. 22, after depletion of these 'bound' stores of Ca^{++}, the addition of Sr^{++} to the solution bathing the muscle restored both IsoKCl-induced and caffeine-induced contractures (Fig. 22-C). After replacing the Sr^{++} with Ca^{++} there was a rapid decline in the tension of IsoKCl-induced contractures but the caffeine contracture tension increased (Fig. 22-D).

In the experiment shown in Fig. 23 the muscle was tested

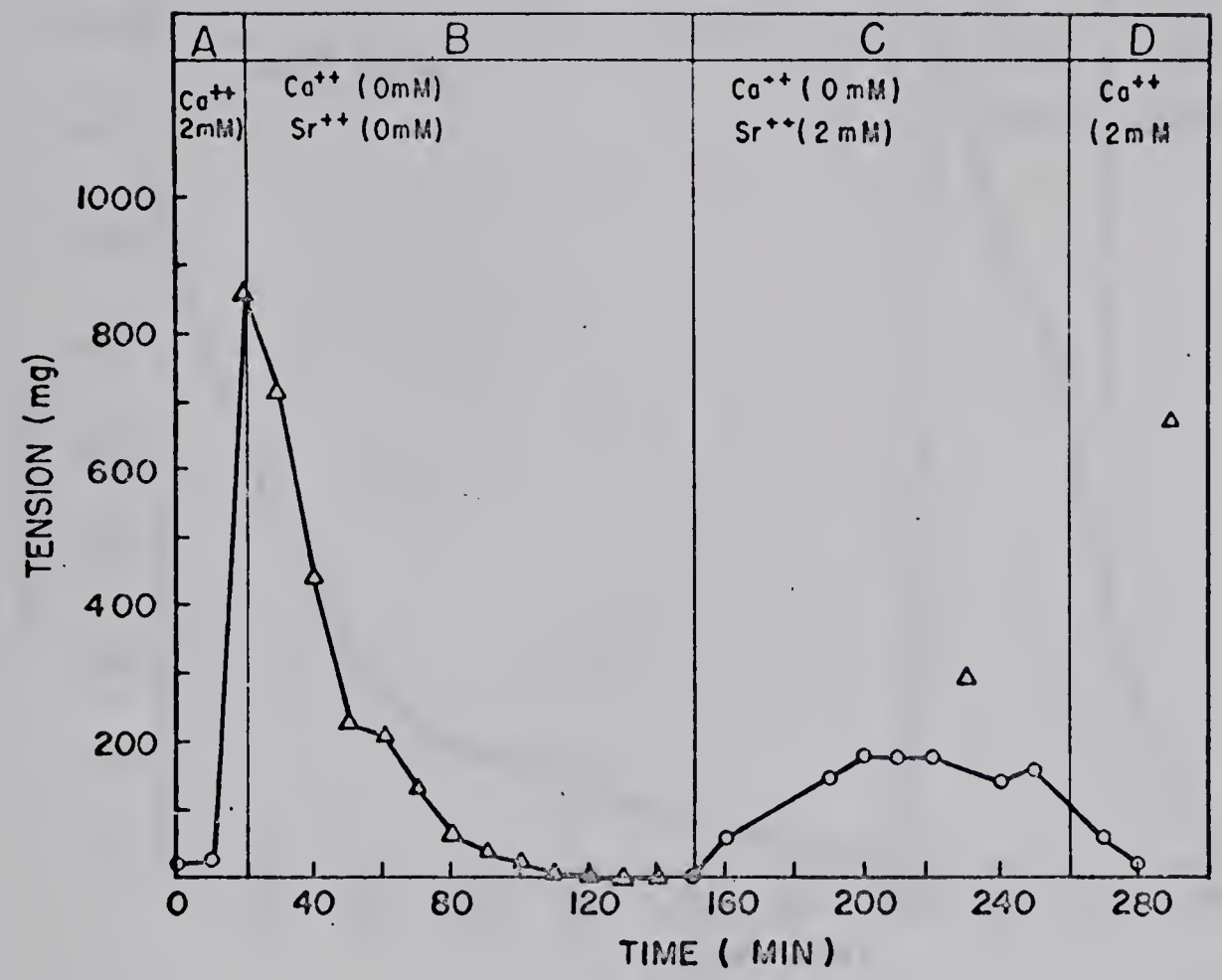

Fig. (22). Isotonic KCl- and caffeine-induced contractures supported by Sr^{++} after Ca^{++} depletion by caffeine in Ca^{++}-free Krebs in the rat lumbricale. (O——O), IsoKCl contractures; (Δ——Δ), (Δ), 20mM caffeine contractures. A; control IsoKCl contractures in Ca^{++} (2mM) Krebs. B; 20mM caffeine contractures eliminated in Ca^{++}-free Krebs. C; IsoKCl and 20mM caffeine contractures in Ca^{++}-free, Sr^{++} (2mM) Krebs. D; IsoKCl and 20mM caffeine contractures in Ca^{++} (2mM) Krebs.

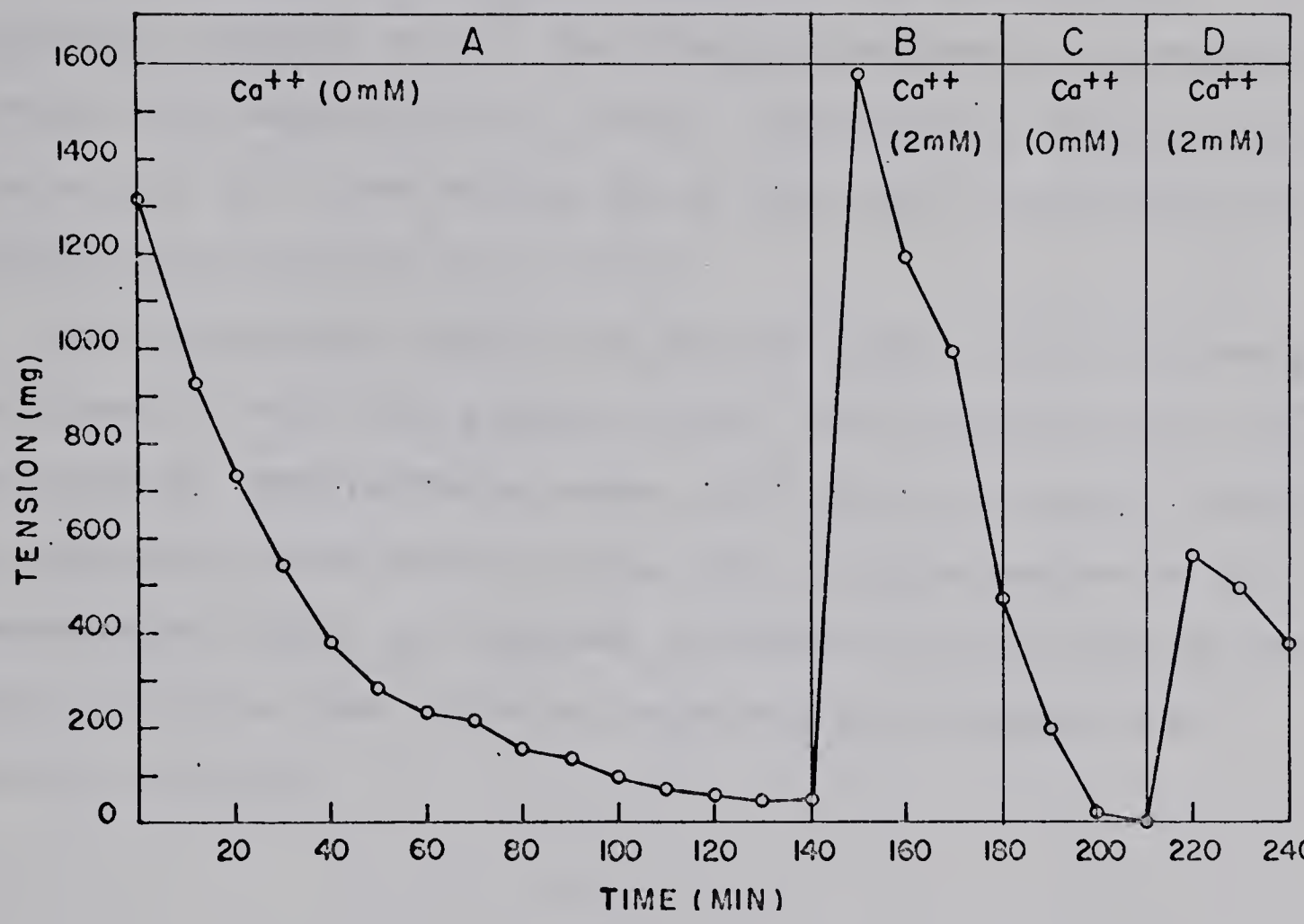

Fig. (23). Control caffeine contractures in Ca^{++} Krebs following depletion in Ca^{++}-free Krebs in the rat lumbricale. A and C, 20mM caffeine contractures eliminated in Ca^{++}-free Krebs. B and D, 20mM caffeine contractures in Ca^{++} (2mM) Krebs.

with 20mM caffeine during a 140 minute exposure to a Ca^{++}-free medium. When the muscle was reexposed to Ca^{++}, the response to caffeine was rapidly restored, but even in the continued presence of Ca^{++} the maximum tensions of subsequent contractures declined (Fig. 23-B). Subsequently placing the muscle in a Ca^{++}-free medium led to the rapid elimination of caffeine contractures (Fig. 23-C).

Two experiments similar to the one shown in Fig. 22 were performed in which the effects of Co^{++} were studied after Ca^{++} depletion by 20mM caffeine tests. Co^{++} did not support IsoKCl- or caffeine-induced contractures. The reintroduction of Ca^{++} demonstrated either no response to IsoKCl or very erratic responses, whereas 20mM caffeine responses were greater than pretest controls.

IV DISCUSSION

IV (A) Caffeine

It is evident that under normal physiological conditions mammalian skeletal muscle is very sensitive to caffeine stimulation. This was demonstrated over the range of caffeine concentrations from 2.6 to 20mM while using bathing and caffeine solutions of various compositions. Under the conditions of lowered temperature (22°C) reported by Gutmann and Sandow (1965), but with continuous oxygenation with 95%O_2-5%CO_2, the sensitivity of mammalian skeletal muscle to both caffeine and elevated K^+ was reduced (Fig. 1). The similarity of the effects on these two responses suggests that a reduction in temperature has a non-specific depressant effect upon mammalian muscle.

When oxygenation of the preparation was stopped after 30 minutes at 22°C, and then testing begun, isotonic KCl-induced contractures rapidly diminished or disappeared while caffeine contractures remained for 5-6 hours of testing. Under these conditions, however, the preparations were strongly influenced by changes in pH (Fig. 2). At lower pH values, the sensitivity to caffeine was greatly depressed and at higher pH values these contractures were greatly increased in size. Under normal physiological conditions the preparations also were sensitive to pH changes, but to a smaller extent. Generally it was necessary to change the pH during caffeine contracture to clearly demonstrate pH-induced effects at 37°C (Fig. 3).

Thus, of the several modifications made in our experimental

conditions, only dissolving the caffeine directly into solution without the aid of HCl and the reductions of temperature and caffeine test solution pH produced a marked reduction in sensitivity to caffeine stimulation. A combination of these factors, however, could lead to the mistaken conclusion that denervation is a necessary prerequisite for caffeine-induced contracture as Gutmann and Sandow (1965) contend. While chronic denervation does not appear to be a necessary prerequisite for the production of caffeine-induced contractures in rat skeletal muscle, it is still possible that chronic denervation renders rat skeletal muscles less sensitive to the changes in the experimental conditions which would ordinarily cause a reduction in the sensitivity of these muscles to caffeine stimulation. On the basis of the work presented here, however, it appears that caffeine responses are qualitatively the same in amphibian and mammalian skeletal muscle systems, and that the structural-functional relationships ostensibly revealed by caffeine may be assumed to be common to both systems.

The variable tendency for caffeine contractures to decrease with repeated exposures to this drug has been ascribed to a functional decline brought about through solubilization of acidic muscle substituents. This is consistent with the fact that caffeine and analogues such as theophylline are often added to organic acids or other compounds to increase solubility, the xanthines forming complexes with the solute. The acidic lipids are believed to supply anionic sites for membrane Ca^{++}

binding (Hauser and Dawson, 1967). If caffeine complexed with these membrane substituents, it is obvious that this could account for the reduction or disappearance of isotonic KCl-induced contractures and electrically stimulated contraction after repeated caffeine stimulation. Competition between Ca^{++} and complex forming caffeine might also occur in the membrane. The organic acids of the membrane may also act as carriers to transport Ca^{++} across the membrane if they complex with caffeine. In addition, solubilization of internal substituents could result in the decreased size of caffeine contracture seen with repeated stimulation.

This work brings up the interesting question of the site of action and the mechanism whereby pH changes influence caffeine-induced contractures. In an initial series of experiments, it was found that there were no consistent effects of pH changes in bathing or test solutions on isotonic KCl-induced contractures, indicating that pH effects are probably not due to any modification of the contractile mechanism itself (Figs. 4 and 5). This finding is in contrast to a recent report by Lorković (1967), which indicates that the concentration of K^+ needed to produce contractures in frog toe muscles increases 3.5 times when the pH is lowered from 7 to 5, but is unchanged on going from 7 to 9. However, the pH range (5 to 7) in which frog muscle is sensitive to pH changes in elevated K^+ solutions is below the pH range (7 to 9) in which mammalian skeletal muscle is most sensitive to pH changes in caffeine solutions.

In addition, the magnitude of changes in contracture tension induced by pH changes in elevated K^+ demonstrated by Lorković in frog skeletal muscle is nowhere near the magnitude of changes in contracture tension induced by pH changes in caffeine in mammalian skeletal muscle. Lorković also reported that caffeine (3mM) contractures in frog toe muscles were not affected by pH when tested at pH's 5 and 7.

The rapid onset of effects due to changes in solution pH on the caffeine-induced contracture suggest that they result from a modification of the caffeine molecule. However, despite these rapid effects, it seems unlikely that the pH dependent modifications of caffeine sensitivity are due to changes in the amount of unionized caffeine in solution. Thus caffeine ionizes only as the cation, and calculations using a pK_a of 0.8 (Turner and Osol, 1949; Schanker et. al., 1957) demonstrate that more than 99.9% of the caffeine remains unionized over the entire range of our study. A similar conclusion was reached by Hardman (1962) in an attempt to explain the increase in positive inotrophic activity of caffeine with increasing pH in the isolated turtle heart. Inotrophic effects induced by solution pH changes using caffeine analogues, however, were explained on the basis of changes in their degree of ionization.

Thus it would appear that the changes in caffeine-induced contracture tensions are due to an action of the pH change upon the caffeine receptor or upon the site of bound Ca^{++}. Axelsson and Thesleff (1958) found that methylated xanthines other than caffeine (e.g. theobromine and theophylline) produced similar

contractures in frog skeletal muscle, but were less potent. Hardman and Reynolds (1965), also found that in turtle heart preparations there were pH effects upon the inotrophic response to ephinephrine that could not be ascribed to drug ionization. Hardman and Reynolds suggested a specific pH effect upon ephinephrine inotrophic receptors.

Guttman and Higuchi (1957) found that the partitioning of caffeine between an organic and aqueous phase suggested that caffeine is markedly concentrated in the aqueous phase as the dimer and tetramer. This self-complexing is not unduly surprising in view of the ability of caffeine to form molecular complexes with a wide variety of organic compounds; including structurally similar xanthine derivatives. Experiments using 7-substituted theophylline indicated a decreasing degree of association in the aqueous phase with increasing chain length. When theophylline was methylated to form caffeine, there was a marked increase in aqueous solubility. This methyl increment in the 7 position, and further lengthening of the side chain, apparently weakened intermolecular forces operating in the crystal lattice, and therefore solubility was increased. A substitution larger than methyl on the N_1 of the basic xanthine nucleus radically reduced the degree of associative ability, blocking e.g., the formation of tetramers. Lengthening of the N_7 substituent did not show as distinct an associative blockade, but did reduce dimer and tetramer formation. Guttman and Higuchi (1957) suggested that steric factors are involved in

both cases and that the associative centers are located in the polar pyrimidine-dione ring of the xanthine. While it is not possible to determine the precise nature of these interactions, they are undoubtedly the same forces involved in the formation of complexes.

Caffeine association was also found by Guttman and Higuchi (1957) to be a function of concentration and temperature. Using conditions which insured adequate aqueous solution (agitation at 30°C for several hours), 20mM caffeine was found to exist as approximately 5mM dimer and 15mM monomer. No tetramer existed at this caffeine concentration and temperature. More complex formed, however, at lower temperatures. Only the monomer was assumed to appear in the organic phase. Undoubtedly there is an equilibrium between monomeric concentration in the aqueous phase and in the organic phase, but an equilibrium between dimer-tetramer and monomer in the aqueous phase may not exist unless adequate solubilization has been achieved. This would reduce the monomeric concentration in the membrane and presumably reduce caffeine effectiveness.

These findings provide a basis for explaining the increased effectiveness of acid-dissolved caffeine. This procedure would insure adequate solubilization and explain the concentration but does not difference seen in the ultraviolet spectrophotometric absorption curves (Fig. 6). Because caffeine self-association is concentration dependent, the 1/200 dilutions of 20mM caffeine used for the ultraviolet curves probably exhibit a spuriously low

concentration difference between acid-dissolved and the directly dissolved caffeine. This caffeine self-association also may be pH dependent once adequate solubilization is achieved. Increased complex formation with lowered temperature might also partially explain the reduced effectiveness of caffeine at lowered temperatures, although there are, of course, temperature effects on the muscle as well. Caffeine molecules also may aggregate as a function of time. This would explain the increased effectiveness of fresh solutions as compared to older solutions.

Bianchi (1962) has shown that caffeine moves essentially freely through frog skeletal muscle, and Isaacson and Sandow (1967) that it moves freely through normal and denervated rat skeletal muscle. Caffeine, although undissociated at physiological pH, is polar and is much more soluble in water than in benzene. It has a benzene-water partition coefficient of 0.04 (Guttman and Higuchi, 1957). Thus if the cell membrane behaved in a fashion similar to benzene, caffeine would penetrate only with difficulty. In contrast, the cloroform-water caffeine partition coefficient is 24. If the organic solvent is somewhat acidic in nature then it appears that a polar compound such as caffeine may be taken up by hydrogen bond formation. "To explain the rapid penetration of caffeine", in the words of Bianchi, "it is tempting to assign hydrogen bonding properties to the cell membrane similar to those of chloroform."

Whether Bianchi is precisely correct or not, the ability of pH changes to influence contracture development may be

a reflection of pH-dependent changes in the membrane which permit faster or slower caffeine penetration. This hypothesis would explain several of the effects of pH on caffeine sensitivity. It would, e.g., explain the rapid onset of pH-produced changes in caffeine contractures and still allow for the action of caffeine to occur internally at the level of bound Ca^{++}. The effect of pH on caffeine sensitivity would thus be due to a caffeine concentration change at an intracellular site, controlled, in turn, by a pH modification of membrane caffeine permeability.

Guttman and Gadzala (1965) studied the aqueous spectral characteristics of xanthine derivatives including caffeine and found them to be changed by the presence of bovine and human serum albumin. The effects observed were interpreted as indicating protein binding of the xanthines. This binding varied as a function of the xanthine used, the protein used, the pH and the temperature. Maximal interaction appeared to require specific orientation of the protein molecule, and expansion or pH-induced configurational changes in the protein resulted in decreases in binding. The pH profile suggested that the protein binding site was a protonated amino group; possibly the epsilon amino group of a lysine residue, and that this site could be neutralized or acetylated with a resultant binding inhibition. Variations of pH above and below the protein isoelectric point exhibited a binding decrease with complete inhibition at pH extremes. Transitions on either side of the isoelectric point

with its attendant binding inhibition are believed due to electrostatic protein expansion brought about by the protonation of protein carboxyl groups. The protein binding site probably contains a hydrogen donor grouping which hydrogen bonds to xanthines at the C_6 oxygen. Caffeine was bound to a weaker extent than the 8-substituted caffeines, and this suggested a strengthened interaction by nonpolar substituents in the 8 position. These findings also suggest that caffeine may be bound to or pulled through the membrane to act at internal sites as a function of external pH.

Because of the speed of caffeine activation, its effects have been described as due to an effect on the external membrane despite the lack of changes in resting membrane potential or in transverse membrane resistance due to caffeine. Changes in pH affecting the responses to caffeine most probably act on the membrane in some fashion, since it has been shown that the molecular species of caffeine is not changed and it is doubtful that external pH changes can have significant or immediate influences on the pH in the intracellular region of the sarcoplasmic reticulum. Changes in pH do produce changes in the electrical conductance and in the production and duration of the action potential in the frog skeletal muscle fiber membrane. Alkali solutions were found to labilize the cation carrying systems to lower the threshold for action potential production. The action potential was also found to be conducted faster and to rise and fall faster. Acidification slowed all phases of the action potential and stabilized the membrane (Brooks and Hutter, 1963).

Adler, Roy, and Relman (1965) used labelled DMO (5,5-dimethyl-2,4-oxazolidine-dione) to measure the steady state intracellular pH changes in rat diaphragm produced by varying CO_2 tensions at a constant bicarbonate concentration, or by varying bicarbonate concentrations at a constant CO_2 tension. Contrary to the prevalent opinion, they found that the pH of muscle cells was readily influenced by both the external bicarbonate concentration and the CO_2 tension. With alkalinization, which the muscle cells resisted much less effectively than acidification, increases in Na^+, K^+, and water also occurred. Intracellular pH followed the external pH fairly closely from a pH value of 6.7 to approximately 6.9. At this point cellular pH increased very slowly, remaining near 6.9, until an external pH of approximately 7.4 was reached. At this point cellular values rose sharply with increasing external pH values, although there was a significant difference between the intracellular and extracellular values (e.g., using approximate pH values; extracellular 7.7 and intracellular 7.2).

This method of intracellular pH determination does not show the different H^+ concentrations that might exist locally in different regions of a single cell. Nevertheless, it does suggest that intracellular pH may follow extracellular pH more closely than is currently believed. If this is the case, pH changes in caffeine solutions may be able to act more or less directly at the intracellular level of bound Ca^{++}. It should be pointed out again, however, that Adler et. al., (1965)

measured steady state pH values, while the pH dependent changes in caffeine responses generally occurred within a few seconds.

Experiments were performed to test the effects of different pH values on the time course of the elimination of caffeine-induced contractures in a Ca^{++}-free Krebs solution. If caffeine does act upon the bound Ca^{++} site to increase the activity of Ca^{++} as a function of pH, then the more effective higher pH values should lead to a faster depletion of bound Ca^{++} stores. Regardless of the pH used, however, a second elimination of the caffeine sensitivity was always more rapid; possibly as a function of degenerative processes in the muscle which are increased in time by caffeine. This effect is demonstrated in Fig. 23, with the second depletion carried out at the same solution pH as the first.

There are other possible sites of pH-induced changes that could influence caffeine responses. Delcher and Shipp (1966) have reported that alkalosis promoted glycolysis and acidosis decreased glycolysis at the cellular level in the perfused rat heart. One of the intracellular reactions controlling glucose metabolism is the phosphofructokinase reaction which converts fructose-6-phosphate to fructose-1,6-diphosphate. Phosphofructokinase functioned optimally at pH 8.0 and greatly decreased in activity as pH 7.0 was approached. Glucose uptake and lactate production decreased directly with decreased pH from 7.5 to 7.15 and 6.8. Delcher and Shipp (1966) suggested that the decrease

in glucose uptake and lactate production at acidic pH values could reflect changes induced in membrane transport, glucose phosphorylation or phosphofructokinase activity. One major unresolved problem is, of course, how strongly extracellular pH changes are reflected at local intracellular sites and how strongly these changes influence intracellular enzyme systems. Trivedi and Danforth (1966) have shown that phosphofructokinase from frog and mouse skeletal muscle is sensitive to small pH changes in the physiological range. These workers note that pH changes do occur within muscle cells and that these changes affect glycolysis.

Both catecholamines and caffeine augment the activity of the phosphorylase enzyme system which initiates the metabolism of glycogen (Sutherland and Rall, 1960). One of the main reactions in this system, the activation of the relatively inactive phosphorylase b to active phosphorylase a by phosphorylase-b-kinase, is pH sensitive (Krebs et. al., 1964). Barboriak and Hardman (1966) have suggested that this enzyme system is the one responsible for the suppression of glycogenolysis with lowered pH in the turtle heart. Danforth and Helmreich (1964) and Meyer et. al., (1964), have shown that Ca^{++} can serve as an activator of phosphorylase-b-kinase, although chelating compounds will not subsequently inactivate the enzyme. Cyclic 3',5'-AMP may activate phosphorylase a and caffeine is believed to act by inhibiting 3',5'-nucleotide phosphodiesterase, which converts 3',5'-AMP to 5'-AMP. It is evident

that this enzyme system could be one site of pH-induced changes in caffeine sensitivity, and should be investigated further for this possibility.

Further experiments could be projected which may shed light on the site of caffeine action. Studies should be performed on the ability of caffeine to accumulate in and to permeate films of membrane substituents (e.g. lecithin) as a function of pH. Numerous experiments could also be performed on isolated sarcoplasmic reticular vesicle preparations. Thus it would be worthwhile to study the flux of Ca^{++} from isolated sarcoplasmic reticular vesicles under the influence of caffeine at various pH's, or to try intracellular injections of caffeine at different pH's to study the effects on contracture. Using experiments of this nature, it may be possible to infer whether it is the intracellular concentration of caffeine or intracellular pH changes which influence contracture producing ability. The former possibility would argue for pH-induced membrane changes, the latter for pH-induced changes at the site of Ca^{++} binding. It is possible, however, that both of these changes will influence the activity of Ca^{++}.

Thus it appears evident that mammalian skeletal muscle is sensitive to caffeine-induced contractures and that these contractures are similar to those that have been produced in frog skeletal muscle. It is reasonable, therefore, to assume that the structural-functional relationships which are believed to be revealed by caffeine are similar in both skeletal muscle systems. For example, it appears from these experiments that

caffeine produces contractures by mobilizing bound Ca^{++} in mammalian skeletal muscle as it has been shown to do in frog skeletal muscle.

In mammalian skeletal muscle, caffeine contractures are pH dependent and are reduced or eliminated at lower pH values (6.0-6.5), and very large at pH values of 7.8 and above. The pH dependence of caffeine contracture is not due to changes in the concentration of unionized caffeine in solution, but probably represents a pH sensitivity of the caffeine receptor or the site of bound Ca^{++}. This pH dependence could reflect a pH dependent membrane passage of caffeine, permitting it to act at internal sites as a function of external pH. Evidence for this possibility is presented in the finding of pH dependent binding of caffeine to proteins. The mechanism whereby pH influences caffeine- induced contractures, however, remains unresolved.

IV (B) Ion substitution experiments

Just as in the case of frog muscle, soaking the rat lumbricale muscle in a Ca^{++}-free solution for several minutes can eliminate IsoKCl-induced contractures (Figs. 7 and 8). In contrast to frog muscle, however, bathing the rat skeletal muscle in a Ca^{++}-free medium and testing with IsoKCl obviously has some deleterious effect; following an initial test of this type there is a subsequent recovery of contracture tension to only 60-70% of control values, and the recovery of contracture tension follows a slower time course than the loss of tension in

a Ca^{++}-free medium. These effects are very similar, however, whether the lumbricale is bathed in a Ca^{++}-free Krebs solution and tested with IsoKCl every 10 minutes for 20 minutes or for 2 hours. If the recovery data is plotted as % of maximum recovery contracture tension (dotted line, Fig. 7), then the slope of the recovery curve more closely resembles that of the contracture elimination curve. In this sense, the lowered recovery curves depicted in Fig. 7 are at least in part an artefact of plotting. When the contracture elimination in a Ca^{++}-free medium is repeated a second and third time (Fig. 8), the recovery curve more closely approximates the contracture elimination curve with each successive contracture elimination. This effect could be accounted for by assuming that a Ca^{++}-free medium has some constant, inactivating effect upon the muscle such as the irreversible inactivation of a certain population of muscle enzymes which optimize the contracture response to IsoKCl. This effect could also reflect an equilibrium reached by the isolated muscle preparation in oxygenated Krebs as a function of time. However, it would seem that immersion in a Ca^{++}-free medium must potentiate this depressant effect of time, since it could be expected that the comparable (in time) equilibrium tension values in a medium containing Ca^{++} would be 85-95% of control.

The slow recovery of IsoKCl sensitivity is difficult to account for. If Ca^{++} diffuses outwards from a binding site on the muscle fibers, then it might be expected that contracture elimination in a Ca^{++}-free medium would take longer than recovery in a solution with Ca^{++} rather than the reverse.

From the experiments performed, it is not possible to determine whether the cations tested with IsoKCl exert an effect on the membrane or on some internal site such as the sarcoplasmic reticulum, or both.

Sandow, Taylor and Preiser (1965) and Sandow and Isaacson (1967) have shown that twitch potentiation is produced by many divalent metallic cations. This potentiation could be correlated with a prolongation of the action potential. Since the potentiating effects occurred via the action potential, and the effects of the divalent metallic cations could be rapidly reversed by non-penetrating agents such as EDTA, Sandow and Isaacson (1967) concluded that the divalent cation potentiators exerted their primary effects at external membrane sites. Some of these metallic potentiators, e.g. the highly effective UO_2^{++}, are known to form large polynuclear complexes with a high charge, and thus it would seem most reasonable to assume that they act externally (Ahrland, et. al., 1954).

Frank (1962) suggested that the most likely site of action of the divalent metallic cations in supporting IsoKCl-induced contractures in Ca^{++}-free solutions was the site of bound Ca^{++}. The eventual loss of IsoKCl-induced contractures supported by the divalent cations would then be due to the gradual loss of Ca^{++} from this bound site. He supported this hypothesis by interaction experiments with caffeine, which apparently acted at the same site as the divalent metallic cations by releasing Ca^{++}.

Lorković (1967) found that Co^{++}, Ni^{++} and Mn^{++} increased

the mechanical threshold (i.e., the degree of depolarization required to initiate a mechanical response) and K^+-induced contracture size in frog toe muscles, while Mg^{++} and Sr^{++} decreased the mechanical threshold and the K^+-induced contracture curve area while maintaining approximately the same tension as that induced by Ca^{++}. Cd^{++} reduced, and Zn^{++} did not change the mechanical threshold, but both increased the contracture curve area and the maximum contracture tension induced by IsoKCl. When isolated frog sarcoplasmic reticular vesicles were loaded with ^{45}Ca, this Ca^{++} was partially released in the presence of Cd^{++} or Zn^{++}, whereas Ni^{++} and Mn^{++} were not effective. Release of ^{45}Ca was increased from resting whole frog toe muscle preparations by Co^{++}, Zn^{++}, and Cd^{++}. The washout of self-exchangeable Ca^{++} in resting muscle was increased when Co^{++} or Mn^{++} was present, but was an order of magnitude slower than that induced by Ca^{++}.

Lorković recognized the danger of drawing conclusions from his experiments on isolated sarcoplasmic reticulum, but suggested that, in view of the lack of ^{45}Ca release from the vesicles by Mn^{++} and Ni^{++}, it was improbable tht the divalent cations penetrated the membrane from the extracellular medium and released reticular Ca^{++} directly. Lorković then postulated an intermediate process at the membrane level, with an unspecific requirement for divalent cations.

Whereas externally applied Ni^{++} is more effective than externally applied Sr^{++} (Frank, 1962; Lorković, 1967), the opposite

effect is seen in the skinned fiber (Edwards, Lorković and Weber, 1966). This seems to imply that the external cations are not directly involved in contraction. The role of the external cations may be, then, the maintenance of an undefined cell membrane property (Jenden and Reger, 1963). One possibility is that the divalent cations might be unspecifically required to induce a phase transition (e.g. oil-in-water to water-in-oil) in the membrane with excitation. Such transitions are known to be governed in some cases by the univalent-divalent ion ratio (Tasaki and Singer, 1966). The possibility remains that selective membrane permeability barriers exist which might explain the discrepencies between internally and externally applied Ni^{++} and Sr^{++}. Lorković's findings of Ca^{++} release from whole muscle and from sarcoplasmic reticular vesicles, however, are suggestive of Ca^{++} release to support K^{+}-induced contracture in spite of Lorković's own reservations. It is possible that these cations act by combining varying degrees of membrane and internal action which cannot yet be properly differentiated.

Lorković (1967) retained the hypothesis of the direct action of Cd^{++} on the sarcoplasmic reticulum to explain lowering of the mechanical threshold. A slower reabsorption of Ca^{++} under the influence of Cd^{++} or Zn^{++} in the intact muscle could explain the prolongation of contractures seen with these cations. Carvalho (1966) found that fragmented sarcoplasmic reticulum from rabbit skeletal muscle bound $H^{+} >> Zn^{++} > Ca^{++} > Mg^{++} >> Na^{+}=K^{+}$. He found this binding to depend upon a cation

exchange type of interaction. This finding suggests that divalent metallic cations do bind to the mammalian sarcoplasmic reticulum and cation exchanges occur.

Hagiwara and Takahashi (1967) reported the effects of various cations in the external solution upon the Ca^{++} spike of the barnacle muscle membrane. Their analysis of the rate of spike potential rise indicated that the divalent cations bind to the same membrane sites competitively. This binding occurred in the order Zn^{++}, Co^{++}, Fe^{++} > Mn^{++} > Ni^{++} > Ca^{++} Mg^{++}, Sr^{++}. Sr^{++} and Ba^{++} could restore the Ca^{++} spike of the barnacle muscle fiber in a Ca^{++}-free medium, whereas, in contrast, the other divalent cations acted as inhibitors. Sr^{++} and Ba^{++} have also been found to restore transmitter release in a Ca^{++}-free medium at the neuromuscular junction (Elmqvist and Felman, 1965; Miledi, 1966). Daniel (1963) found Sr^{++} and Ba^{++} capable of maintaining drug-induced contractures in a Ca^{++}-free medium in certain smooth muscle preparations, although the effects of these two cations differed in some respects.

In a large number of cardiac muscle preparations (e.g. from cat, rat and frog), the inotrophic effects of Ca^{++} may be partially duplicated by Sr^{++} (Weyne, 1966). Weyne found a positive inotrophic effect when Sr^{++} was substituted for Ca^{++} in the perfusion medium. The contraction was stronger, but slower and longer in a Sr^{++} medium (Hemtinne, Weyne, and Leusen, 1967).

The effects of the divalent metallic cations obtained in the present experiments can probably be best summarized in the form of a table:

TABLE I

Effects of divalent metallic cations on IsoKCl contractures of rat lumbricales.

Divalent cation	Responses in solutions with OCa^{++} with the cation	Ca^{++} with the cation	Ca^{++} after cation removal
Mg^{++} (1)	None	Tonic $<<$ control (a)	Tonic or phasic-tonic $<<$ control $>Mg^{++} + Ca^{++}$ responses
Ba^{++} (2)	Tonic, slow, often of appreciable size	Tonic, slow $<<$control $>Ba^{++}$ responses	Phasic or phasic-tonic $<$control $>Ba^{++}+Ca^{++}$ responses
Ni^{++} (3)	Tonic, slow, small	Tonic, slow $<$control $>Ni^{++}$ responses	Phasic $>>$control (b)
Co^{++} (4)	Transiently phasic, small then tonic	Phasic without relaxation $\geq$control (c) $>>Co^{++}$ responses	Phasic, erratic $>>$control $>$ $Co^{++}+Ca^{++}$ responses
Sr^{++} (5)	Phasic $\geq$ Control	Phasic $\geq$control (d) $<Sr^{++}$ responses	Phasic $<$control $<Sr^{++}+Ca^{++}$ responses

(1) Figs. 9,10; (2) Figs. 11,12; (3) Figs. 13,14,15; (4) Figs. 16,17,18; (5) Figs. 19,20,21.

(a) Control in all cases refers to recovery to 60-70% original tension (see Figs. 7,8).

(b) Also obtained without exposure to OCa^{++} with Ni^{++}.

(c) Co^{++} potentiates after an initial depression when added directly to Ca^{++} and delays contracture elimination in OCa^{++} medium.

(d) Sr^{++} will maintain potentiated responses in OCa^{++} for more than 5 hours (see Fig. 21), i.e. is as effective as Ca^{++} or more so.

(e) $Sr^{++}+Ca^{++}$ from Ca^{++} maintains responses well at approximately double control values.

Frank (1962) and Lorkovic (1967) found Mg^{++} to support IsoKCl-induced contractures in frog toe muscles in a Ca^{++}-free solution, but less effectively than Ca^{++}. In contrast, Mg^{++} was incapable of supporting IsoKCl-induced contractures in mammalian skeletal muscle and exerted a lasting depressant effect which was at least partially dependent upon the length of time the muscle remained in a Ca^{++}-free, elevated Mg^{++} Krebs solution.

Frank (1962) found Ba^{++} incapable of supporting IsoKCl-induced contractures in frog toe muscles in a Ca^{++}-free solution. In contrast, Ba^{++} was found to support a variable, slow, tonic contracture in mammalian skeletal muscle. Contractures remained variable in size, tonic, and slow with the addition of Ca^{++} to the solution with Ba^{++}, but the contracture tensions approached values near control when Ba^{++} was removed from the medium. These contractures varied in form between tonic and phasic responses, and thus even after its removal Ba^{++} may be assumed to show continuing effects to various degrees.

Frank (1962) and Lorkovic (1967) found Ni^{++} to support K^{+}-induced contractures larger than those supported by Ca^{++}. In contrast, in mammalian skeletal muscle, Ni^{++} supported only tonic, slow, small contractures. These remained similar in shape, but were transiently increased when Ca^{++} and Ni^{++} were added together. Transiently potentiated phasic contractures followed the removal of Ni^{++} from the bathing solution. This potentiating effect was also seen when the preparation was placed in a Krebs containing Ca^{++} from one containing both Ca^{++} and Ni^{++} without a previous test with Ni^{++} in a Ca^{++}-free

Krebs solution.

These variable, tonic responses in the presence of Ba^{++} or Ni^{++} suggest a low-level Ca^{++} activation or Ca^{++} displacement from intracellular sites. The possibility remains, however, that Ba^{++} and Ni^{++} may be partially capable of substituting for Ca^{++} at a membrane site. Ba^{++}, Ni^{++} and Mg^{++} all inhibit the effects of Ca^{++}, although the presence of Ca^{++} in the bathing solution can antagonize the effects of these cations to some extent. This effect may be caused by a decreased Ca^{++} permeability of the membrane caused by the presence of these cations on membrane anionic sites. Although Ba^{++} and Mg^{++} maintain an inhibitory effect after washout, Ni^{++} washout potentiates the subsequent IsoKCl contractures induced in a Krebs solution containing Ca^{++}. It is possible that low Ni^{++} concentrations potentiate the IsoKCl response, but it is also possible that some active site is changed by the presence of Ni^{++} since the potentiated responses return to phasicity from the tonic responses seen in the presence of Ni^{++} in the bathing solution.

Frank (1962) and Lorković (1967) found Co^{++} more effective than or equivalent to Ca^{++} in supporting ISoKCl-induced contractures in frog toe muscles. In mammalian skeletal muscles, Co^{++} was found to support phasic contractures for 50 minutes, after which low, tonic responses were obtained. When Ca^{++} and Co^{++} were subsequently introduced together, contracture tensions were very close to control values, but the effects of Co^{++} were still in evidence as shown by contractures which were

somewhat slow in developing tension and had no clear relaxation phase. Phasic, potentiated responses occurred when the muscles were returned to a normal Krebs, but these responses were erratic in shape. When the preparation was placed in a Ca^{++}, Co^{++} Krebs from normal Krebs , a transient potentiation occurred after an initial decreased response, and occurred again to lesser extent when the medium was changed back to Krebs with Ca^{++}.

After transition from the phasic to the tonic Co^{++} supported IsoKCl-induced contracture, 20mM caffeine induced good caffeine responses which relaxed more slowly than usual. It is obvious that the short time required to eliminate the phasic Co^{++} supported response in Ca^{++}-free, Co^{++} Krebs was not sufficient to deplete the bound Ca^{++} store. When Ca^{++} replaced Co^{++} the 20mM caffeine response was potentiated, but also relaxed slowly. The absence of a clear relaxation in the IsoKCl-induced contracture in the presence of Ca^{++} and Co^{++}, and in the caffeine contracture in the presence of Co^{++}, suggests that Co^{++} inhibits the reaccumulation of Ca^{++} by the sarcoplasmic reticulum. When caffeine responses were eliminated by repeated exposure to 20mM caffeine in a Ca^{++}-free Krebs solution, Co^{++} was not capable of supporting IsoKCl contractures.

Co^{++}, then, is capable of supporting IsoKCl-induced contractures for a short time and appears to transiently potentiate the effects of Ca^{++} when these cations are added together. This transient period is approximately of the same duration in both

instances (i.e., Co^{++} alone or Co^{++} with Ca^{++}). From these findings it appears that Co^{++} may exert a potentiating effect on IsoKCl contractures at low concentrations and an inhibitory effect at higher concentrations. The transient potentiation seen with Co^{++} when added to a Krebs containing Ca^{++} would then reflect the time required to build up an appreciable auto-inhibitory concentration of Co^{++} at an active site (Fig. 18-B). This hypothesis is suggested further by the initially depressed IsoKCl contracture before Co^{++} potentiation in Fig. 18-B, the potentiated IsoKCl responses after Co^{++} washout seen in Fig. 18-C, and the similar time courses of Co^{++} potentiation. Interaction experiments with caffeine suggest that Co^{++} acts at a site of Ca^{++} binding to release Ca^{++}, but is not very effective in doing so.

Frank (1962) and Lorković (1967) found Sr^{++} to be similar to Mg^{++} in being less effective than Ca^{++} in supporting IsoKCl-induced contractures in frog toe muscles. In contrast, Sr^{++} was the only ion which demonstrated a clear ability to substitute for Ca^{++} in supporting both IsoKCl- and caffeine-induced contractures in mammalian skeletal muscle. When the preparation was transferred from a Ca^{++} to a Ca^{++}, Sr^{++} medium, the contracture tension doubled and remained near this value. Upon transfer from a Ca^{++} to a Sr^{++} medium the response tension remained near control values, but gradually increased with time. In these instances, Sr^{++} seemed to have an additive effect with Ca^{++}.

The Sr^{++} supported IsoKCl-induced contractures obtained after contracture elimination in a Ca^{++}-free Krebs solution were phasic, and responses could be obtained for longer than 5 hours in the absence of Ca^{++}. These responses were very regular and greatly potentiated compared to those seen with Ca^{++}. As testing continued over the 5 hour period, the responses remained phasic, but developed a long, slow, secondary and relaxation phase. This finding is consistent with the experiments of Edwards, Lorković and Weber (1966), which demonstrated that the sarcoplasmic reticulum took up Sr^{++} (but not Co^{++}), and did so less effectively than Ca^{++} in frog muscle. They also demonstrated that Sr^{++} fully activated myofibrillar ATPase, but the affinity of ATPase for Sr^{++} was only 1/30 of its affinity for Ca^{++}. The increased response size in the presence of Sr^{++} as compared to that in Ca^{++} demonstrated here suggests the possibility of an inverse situation; certainly experiments similar to those of Edwards et. al.. (1966) should be repeated in mammalian skeletal muscle. Sr^{++} also appears unique in that its effects may be overcome or antagonized by Ca^{++}, and Sr^{++} removal leaves subsequent Ca^{++} supported responses relatively unaffected as compared to controls. This unique combination of effects suggests that Sr^{++} may act at the level of the sarcoplasmic reticulum, whereas the other cations may not be capable of effective action at this site.

When the Ca^{++} binding site was depleted by repeated exposures to 20mM caffeine in a Ca^{++}-free solution, Sr^{++} was

found capable subsequently of supporting IsoKCl-induced contractures and 20mM caffeine contractures. The caffeine contracture, however, was smaller than control values. When Ca^{++} was reintroduced subsequently, the IsoKCl-induced contractures were much smaller than the Sr^{++} supported IsoKcl-induced contractures, whereas the 20mM caffeine response doubled to closely approach control values. Both the Sr^{++} and Ca^{++} supported caffeine responses relaxed very slowly, suggesting that the Ca^{++} supported caffeine response may have been affected by a low, remaining Sr^{++} concentration. This is in contrast to the relatively unaffected IsoKCl-induced contractures seen after Sr^{++} removal. It is very interesting that Sr^{++} potentiates IsoKCl-induced responses, but inhibits the caffeine-induced responses. Further work on this finding may point up definite differences in sites of action in these 2 processes. Thus, of the 5 ions tested, only Sr^{++} exhibited a clear ability to substitute for Ca^{++} in supporting excitation-contraction coupling in mammalian skeletal muscle.

IV (C) Comparison of excitation-contraction coupling in amphibian and mammalian skeletal muscle

It is apparent from the experiments described in this thesis that definite parallels, with some differences in the details, may be drawn in the processes of excitation-contraction coupling in amphibian and mammalian skeletal muscle systems.

In both types of muscle a relatively brief soak in a Ca^{++}-free solution results in the elimination of the IsoKCl-induced

contractures. This procedure, however, does not deplete the store of bound Ca^{++}. Caffeine acts in a similar fashion in both muscles to mobilize the bound Ca^{++} store and thus induce contracture. Many of the divalent metallic cations also act on this store of bound Ca^{++} to release Ca^{++} under the influence of IsoKCl, and are thus capable of restoring IsoKCl-induced contractures after their elimination in a Ca^{++}-free medium.

There are some differences, however, in the actions of these divalent metallic cations in supporting IsoKCl-induced contractures in the two muscle systems. Mg^{++}, which is effective in frog muscle, was not capable of supporting IsoKCl-induced contractures in mammalian muscle. Conversely, Ba^{++} was ineffective in frog muscle, but supported IsoKCl-induced responses in mammalian muscle. In frog muscle, Ni^{++} and Co^{++} supported phasic IsoKCl-induced contractures, whereas, in contrast, they gave transiently phasic or tonic responses in mammalian muscle. Sr^{++}, which was intermediate in K^{+}-induced contracture effectiveness in frog muscle, was unique in clearly substituting for Ca^{++} in supporting both IsoKCl- and caffeine-induced contractures in mammalian muscle.

In conclusion, the process of excitation-contraction coupling, as revealed by the action of caffeine and the ability of the divalent metallic cations to support IsoKCl-induced contractures by releasing bound Ca^{++}, is essentially the same in both amphibian and mammalian skeletal muscle systems. While there are differences in the details of the process, these are no more than can be expected in view of the vast differences in the two species.

V SUMMARY AND CONCLUSIONS

1. Caffeine in concentrations between 2.6 and 20mM readily induced contractures of isolated rat skeletal muscles in 37°C, oxygenated sodium Krebs with $NaHCO_3$ or Tris buffer or choline Krebs buffered with $NaHCO_3$. These contractures were influenced by pH changes, but this effect was clearly demonstrated only at such pH extremes as 6.5 and 8.0.

2. Reduction of the temperature from 37° to 22°C with continuous oxygenation reduced both the isotonic KCl- and caffeine-induced contractures.

3. Conditions of 22°c without continuous oxygenation led to a rapid failure of isotonic KCl-induced contractures, while caffeine contractures persisted and were markedly pH dependent. Contractures were very small or absent at pH 6.0-6.5 and very large at pH 7.8 and above.

4. The pH dependence of caffeine contractures is not due to changes in the amount of unionized caffeine in solution, but is probably due to an action upon the caffeine receptor or the site of bound Ca^{++} on which caffeine is believed to act.

5. This pH dependence could reflect a pH dependent membrane passage of caffeine, permitting it to act at internal sites as a function of external pH. This differential ability of the membrane to permit caffeine passage would then have to come strongly into effect only with lowered temperature and incomplete oxygenation.

6. Since caffeine contractures appear to be qualitatively the same in both amphibian and mammalian muscle systems, it is reasonable to assume that the structural-functional relationships which are believed to be revealed by caffeine are similar in both skeletal muscle systems.

7. Elevated Mg^{++} was not capable of supporting isotonic KCl-induced contractures when it was substituted for Ca^{++} in rat skeletal muscle which was continuously oxygenated at 37°C. Mg^{++} exerted a lasting depressant effect on the isotonic KCl response both with Ca^{++} and with Ca^{++} after the removal of excess Mg^{++}.

8. Ba^{++} gave rise to a variable, tonic response by itself and in combination with Ca^{++}, and exerted some lasting depressant effect after its removal.

9. Ni^{++} also gave rise to a variable, tonic response by itself and in combination with Ca^{++}, but potentiated isotonic KCl responses after its removal.

10. Co^{++} gave transient, phasic responses which rapidly became tonic. Co^{++} did not suppress contracture with Ca^{++}, but affected the response as was seen by the absence of a clear relaxation in isotonic KCl. Co^{++} potentiated Ca^{++} supported isotonic KCl-induced contractures after its removal. Co^{++} did not support caffeine contractures after the depletion of bound Ca^{++} in the muscle by repeated caffeine tests in a Ca^{++}-free medium.

11. Sr^{++} was the only ion which demonstrated a clear ability to substitute for Ca^{++}. Sr^{++} maintained phasic isotonic KCl-induced contractures well above control Ca^{++} responses in amplitude. Sr^{++} was also unique in that it did not greatly affect the Ca^{++} supported responses when Ca^{++} and Sr^{++} were added together, and the removal of Sr^{++} seemed to leave subsequent Ca^{++} supported isotonic KCl-induced contractures relatively unaffected. Sr^{++} supported caffeine-induced contractures after the depletion of bound Ca^{++} in the muscle by repeated caffeine exposure in a Ca^{++}-free medium, but was less effective than Ca^{++} in doing so.

12. Sr^{++} appears to be capable of acting itself at internal sites, but its effects may be overcome by the addition of Ca^{++}. The other cations, notably Co^{++}, are apparently capable only of various degrees of Ca^{++} displacement from a binding site and exhibit various effects upon the addition of Ca^{++} to the solution or after removal of the cation. The divalent metallic cations exhibited different degrees of bound Ca^{++} mobilization in frog skeletal muscle.

13. The process of excitation-contraction coupling, as revealed by the action of caffeine and the ability of the divalent metallic cations to support IsoKCl-induced contractures by releasing bound Ca^{++}, is essentially the same in both amphibian and mammalian skeletal muscle systems.

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